ABSTRACT

LI, YOU. Improvement of Food Handling, Sanitation and Hygiene in the Child Care Environment in North Carolina and South Carolina. (Under the direction of Dr Lee-Ann Jaykus).

About 60% of U.S. children, age 5 and younger, spend time in organized child-care. The close and frequent personal contact between children in child-care settings provides many opportunities for the spread of pathogens, particularly those that cause enteric and respiratory diseases. In fact, there is compelling evidence supporting higher illness rates for children cared for in child-care settings. This dissertation represents work on a broad project, the purpose of which was to identify risk factors associated with potential sources of enteric infection in the child-care environment. The focus was on analyzing environmental samples for various microbiological indicators, and to use that data to determine if there were statistically significant associations between microbiological load and child-care facility characteristics, practices, and written procedures.

The first part of this dissertation was a comprehensive literature review summarizing previous studies on hygiene practices in child-care settings. Interestingly, very few of these studies combined microbiological data with observational/survey-based work, providing impetus for this project. In the first research study, environmental samples (corresponding to high touch surfaces and the hands of caretakers) were taken from representative child-care centers located in North and South Carolina. These were analyzed for total aerobic bacteria (APC), coliforms, and generic *Escherichia coli*, as well as for *Shigella* species, *Salmonella enterica*, *E. coli* O157:H7, *Campylobacter jejuni*, and human norovirus. A total of 40 facilities were visited, and 326 samples were evaluated: 74 hand rinsates, and 252 environmental samples. For hands, median APC and coliform counts were 4.5 and 1.8 $\log_{10}$

CFU/hand, respectively. For common surfaces, median APC was 2.5 $\log_{10}$ CFU/surface, with median coliform counts of 1.3 $\log_{10}$ CFU/surface. Generic *E. coli* counts were below the assay detection limits (<1 $\log_{10}$ CFU per sample) for all samples except one hand sample. No samples were positive for any of the four bacterial pathogens, and 7 samples (2.1%) were presumptively positive for norovirus genogroup I, 41 samples (12.6%) were presumptively positive for norovirus genogroup II. The relative absence of pathogens and generic *E. coli* in these samples suggests that the child-care facilities managed fecal contamination well.

In the second study, statistical analysis was used to determine if there were significant relationships between the microbiological data and certain child-care practices, the latter of which were previously determined by a combination of survey and observation. Because much of the microbiological data was above or below assay detection limits, the nonparametric Mann-Whitney U and Kruskal-Wallis tests (p<0.05) were performed to infer differences between two or more populations. Of the 40 child-care facilities studied, 9 were homes and 31 were centers. Samples collected from homes showed significantly higher coliform counts across all surfaces as compared to child-care centers. When comparing indicator levels in accordance with classroom designation [i.e., infants (0-12 months), toddlers (12-36 months), combined (0-36 months), and other (3-5 years], coliform counts on staff hands were significantly higher on samples from infant classrooms than combined classrooms. Mixed results were observed when comparing microbiological indicator levels as a function of provider-to-child ratios. Facilities without a written surface cleaning or food preparation policy had significantly higher APC and coliform counts on surface samples. In general, implementation of written procedures for hygienic practices, and adherence to them, appeared to have a positive impact on overall microbial loads in these facilities. Although

both studies had their limitations (e.g., potential biases, limited sample size, low microbiological indicator counts and the absence of pathogens), they remain unique because they successfully combined observational and microbiological data, providing revealing information on the relationship between hygiene indicators and sanitary practices in child-care facilities in the southeastern United States.

Improvement of Food Handling, Sanitation and Hygiene in the Child Care Environment in North Carolina and South Carolina

by
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DEDICATION

To my parents (Hansheng Li and Jun Jiang)

BIOGRAPHY

You Li was born in Beijing, People's Republic of China. After getting his Bachelor degree on food science at Beijing Agricultural College, he headed Wageningen University (Netherlands) for master study on food safety. Following the procurement of the graduate degree and some internship experience in Dutch, he returned to his undergraduate alma mater to work as an assistant professor teaching food science. From 2009 till now, he is a doctoral candidate at North Carolina State University (US) under the direction Dr Lee-Ann Jaykus.

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Last but not least, it is my parents' love and encouragements that make me to get all the achievements. I am eternally in debt to them.

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LIST OF ABBREVIATIONS

AAP: American Academy of Pediatrics

AOAC: Association of Analytical Communities

AP: Alkaline Phosphatase

APC: Aerobic Plate Counts

APHA: American Public Health Association

BAM: Bacteriological Analytical Manual

BCIP: 5-bromo-4-chloro-3-indolylphosphate

BHI: Brain Heart Infusion

BHQ: Black Hole Quencher

BSA: Bovine Serum Albumin

CACFP: Child and Adult Care Food Program

CDC: Centers for Disease Control and Prevention

CI: Confidence Interval

CFU: Colony Forming Unit

CPR: Cardiopulmonary resuscitation

Ct: Cycle threshold

DEPC: Diethylpyrocarbonate

DIG: digoxigenin

DNA: Deoxyribonucleic acid

df: degree of freedom

dNTP: Deoxyribonucleotide triphosphate

E. coli: Escherichia coli

ELISA: Enzyme Linked Immunosorbent Assay

FAM: 6-carboxyfluorescein

FDA: Food and Drug Administration

GI: gastrointestinal

GI norovirus: genogroup I norovirus

GII norovirus: genogroup II norovirus

IAC: Internal Amplification Control

ICEP: Infection Control Education Program

IRB: Institutional Review Board

IRR: Incidence Rate Ratio

NAEYC: National Association for the Education of Young Children

NARA: National Association for Regulatory Administration

NBT: Nitroblue Tetrazolium Salt

NCCITAC: National Child Care Information and Technical Assistance Center

NC ENR: North Carolina department of Environmental and Natural Resources

NoV: norovirus

ORF: Open Reading Frame

PBS: Phosphate Buffered Saline

PEG: polyethylene glycol

qPCR: quantitative real time Polymerase Chain Reaction

RNA: Ribonucleic acid

RSV: Respiratory Syncytial Virus

RT-qPCR: Reservse Transcription quantitative real time Polymerase Chain Reaction

RT-qPCRU: RT-qPCR amplifiable Unit

RV: Rapport-Vassiliadis medium

STEC: Shiga-Toxin producing *Escherichia coli*

TET: tetrachlorofluorescein

TT: Tetrathionate broth

UPB: Universal Pre-enrichment Broth

USDA: United States of Department of Agriculture

WWII: World War II

XLD: Xylose Lysine Deoxycholate

XLT 4: Xylose Lactose Tergitol™ 4

CHAPTER 1 Management of Gastrointestinal Illness in Out-of-Home Child-care Settings: A Review of the Literature

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Introduction

Child care is an integral part of society in the United States. The National Association of Child Care Resource and Referral Agencies (2009) defines child care, or day care, as *"providing care and/or supervision for children and their daily needs, in a home or center setting, for children from one month of age through twelve years."* In 2005, 61% of U.S. children from birth to six years of age were reported to have spent time in non-parental child care (Child Trends Databank, 2005). Children with employed mothers are estimated to spend more time in child care (25 to 33 hour per week) than are children with unemployed mothers (approximately 19 hours per week) (Lynda, 2010).

The close and frequent personal contact between children in child-care settings provides many opportunities for the spread of pathogens, particularly those that cause enteric and respiratory diseases. In fact, there is compelling evidence supporting higher illness rates for children cared for in child-care centers. For example, Bartlett et al. (1985) reported that the incidence of diarrheal illness in infants and toddlers enrolled in child-care centers (42 cases per 100 child per months) was significantly higher than in daycare homes (27 cases per 100 child per months). Lu et al. (2004) estimated that children cared for outside the home are

between 2.3 and 3.5 times more likely to experience an episode of diarrhea compared to those cared for in their own home, presumably because of their exposure to a large number of children. These investigators also reported that children under 18 months of age who attended child care and were covered by the Medicaid program were at the greatest risk for diarrheal illness. According to the Bradley, R.H. (2003), rates of gastrointestinal (GI) illness were higher in children aged 37 to 54 months enrolled in a larger child-care center (one having more than six children), as compared to a smaller facility (one enrolling less than six children). A case-control study conducted by the U.S. Centers for Disease Control and Prevention (CDC) reported that attendance at a child-care facility in which at least one other child had diarrhea was a risk factor for salmonellosis in infants younger than six months of age (Jones et al., 2006). The only potentially disputing evidence comes from a Danish prospective cohort study which did not find a link between child care and increased risk of gastrointestinal illness resulting in hospitalization (Jorgensen, et al., 2008). However, this study did not examine if there was increased risk of non-hospitalized gastroenteritis in these same children.

In addition to the obvious public health impacts of GI illness, cases and outbreaks in young children can be very costly to a child's family and to the child-care facility. Based on their survey of 379 parents of ill children, Carabin et al. (1999) estimated a cost of at least $270 per child per episode of cold, diarrheal, or vomiting symptoms. These costs, which averaged $100 per episode, including medication, physician visits, lost wages for missed work to care for a sick child, costs for alternative care, lost income for the child-care center, and costs associated with implementing infection control measures. Families also were

reported to have paid an additional $100 in alternatives to child care. Chen et al. (2011) estimated a much lower loss of family income than did Carabin et al. (1999), i.e., $28 per household in association with a child-care center closure due to an influenza outbreak. However, Chen and colleagues further estimated the cost to the facility, which was high. Specifically, a three-day closure resulted in additional staff time that totaled 6,573 hours (69 hours per person over a 10-day period), most of which was dedicated to ensuring facility hygiene and answering health status inquiries. Compare this to a normal work week consisting of 35-40 hours per person and it becomes evident that the cost of an enteric or influenza outbreak in a child-care facility can be quite high.

The proper use of infectious disease control measures is the best way to prevent GI illness and reduce associated costs. For example, Duff et al. (2000) evaluated the cost and functionality of a multidimensional infection control program for various infectious diseases in one child-care center. These researchers estimated that the program cost the facility an additional $2,400 annually but the intervention reportedly saved the center $240 per child per year. Based on the Carabin and colleagues (1999) estimated cost of illness per child ($200), the cost of an infection control program would be offset entirely if twelve or more children were to accrue a benefit. Similarly, Ackerman et al. (2001) estimated the impact of an infection control education program (ICEP) in a specialized preschool setting. In the study's baseline year, the mean cost of illness was estimated to be much higher, $1,235 per child, of which 68% and 14% were attributed to productivity losses and physician visits, respectively. In the intervention year (when the ICEP was implemented), the mean cost of illness per child was $615, of which 71% was for productivity losses and 20% for physician visits. In total,

the authors of the study suggested that the reduction in the cost of illness offset the cost of implementing the ICEP.

It is obvious from the above discussion that the cost of GI illness in child-care facilities is high, and that management of such illness is essential to keeping costs down as well as protecting public health. In this paper, we provide a comprehensive review of the transmission of GI illness in formalized child-care settings. As such, we first identify which agents are most likely to be transmitted in these facilities. The supporting observational and microbiological data are thoroughly reviewed in an effort to identify risk factors for enteric disease transmission in child-care environments. Specific mechanisms by which to control disease spread are identified and their efficacy (or lack thereof) further discussed. We conclude with thoughts on how enteric disease transmission in this important sector might be better controlled in the future.

Etiological Agents Causing Gastrointestinal Illness in Child-Care settings

In their comprehensive review of English-language journals and public health records, published between 1996 and 2006, Lee and Greig (2008) identified 75 reported enteric outbreaks representing a total of 946 confirmed cases that occurred in child-care settings (Table 1.1). 93.4% of identified outbreaks (n=75) were attributed equally to bacteria and viruses, whereas more individuals were affected in outbreaks caused by viral agent (1006 out of 1806 total cases). Only 6.6% of reported outbreaks were attributed to parasitic protozoa. A similar study conducted in Denmark reported that, when excluding 222 cases of unknown etiology, 45% of cases (n=161) were caused by bacterial agents and the remaining 55% by viruses (Ethelberg et al., 2006).

Other published studies also support the importance of viral-borne GI illnesses in the child-care setting. For example, Rosenfeldt et al. (2005) identified viruses as the etiological agent in 69% of 98 diarrheal illness cases in Danish child-care centers, with rotavirus causing the vast majority (40%) of these illnesses. In their study of 29 acute gastroenteritis outbreaks in child-care centers in North Carolina between 2005 and 2007, Lyman et al. (2009) reported that 13/29 (45%) were caused by a single virus, including rotavirus group A (17%), norovirus (10%), astrovirus (10%), and sapovirus (7%). In three outbreaks that were studied, the investigators detected multiple viruses. Akihara et al. (2005) also provided evidence that astroviruses, genogroup II human noroviruses, enteric adenovirus, and sapoviruses were responsible for symptomatic and asymptomatic gastroenteritis in infants enrolled in a child-care center in Japan. The study by Akihara et al. (2005) reported that of 88 fecal samples taken from infants with acute GI illness, 51.1% (45/88) were positive for viruses. Of all positive samples, 15.9% (14/88) were positive for astrovirus, followed by norovirus GII (14.8%, 13/88), adenovirus (12.5%, 11/88), and sapovirus (2.3%, 2/88). A study by Ferson et al. (1997) provided evidence that rotavirus was responsible for asymptomatic and symptomatic GI illness in a child-care center in Australia. These investigators reported that 3.6% (59/1,653) samples were positive for rotavirus. Gabbay et al. (1999) reported that 64 children attending a child-care center in Brazil fell ill due to gastroenteritis associated with group C rotavirus. Astrovirus serotype 1 was the etiological agent for 27 GI illnesses in children attending a Brazilian child-care center (Silva et al. 2001). Hepatitis A virus was also reported by McFarland et al. (2001) as the etiological agent for twelve child GI illness cases in one primary school and one nursery school in the United Kingdom.

A summary of GI illness outbreaks (n=17) in U.S. child-care centers that were published in the scientific literature between 2006 and 2010 is presented in Table 1.2. Of these outbreaks, 41% were caused by *E. coli* O157: H7, resulting in 103 cases. The rest of the cases were caused by various etiological agents (e.g. norovirus, Cryptosporidium, *Shigella spp*). Transmission routes were identified in eleven outbreaks. The fecal-oral route was associated with six outbreaks, non-fecal person-to-person transmission caused four outbreaks, and contaminated recreational pool water was responsible for one outbreak. In addition, a summary of outbreaks reported to CDC FoodNet is provided in Table 1.3. From 1998 to 2008, 51 foodborne disease outbreaks were reported to have occurred in child-care centers. Bacteria were the etiological agent of 28 of these outbreaks (causing 1286 cases) and viruses caused nine outbreaks (with 337 associated cases) due to its detection methods has not been well developed by that time. The etiological agents for the remaining fourteen outbreaks were unknown. The implicated foods fell mainly in the fruit, vegetable, poultry and meat commodity groups.

In our review of the literature, we were unable to find evidence of any deaths of children associated with GI illness definitively transmitted in the child-care setting. However, with respect to hospitalizations, Lee and Greig (2008) reported that for the 75 outbreaks they reviewed, there were a total of 104 total hospitalizations. Seventy -three (73) of these were caused by known bacterial agents, the majority of which were pathogenic *E. coli* strains: 40 cases were caused by *E. coli* O157:H7, 24 cases were caused by *E. coli* O126:H11). Five hospitalizations were caused by *Shigella* and four were associated with *Salmonella*. A total of 29 hospitalizations were associated with illness caused by viral agents

[echovirus (13 cases); rotavirus (8 cases); multiple viral agents (6 cases); Hepatitis A virus (2 cases)]. While viral agents are a more common cause of GI illness in child care, bacterial agents result in more cases requiring hospitalization.

Risk Factors for Pathogens Transmission in Child-Care Settings

In their comprehensive review, Lee and Grieg (2008) identified that person-to-person contact as the most common transmission route for enteric pathogens, responsible for 43% of outbreaks associated with bacterial agents and 60% for parasitic agents. The transmission route for viral agents was largely unknown (51%), but when identified, person-to-person transmission (40%) was most common. Contaminated food was the next most common transmission route, responsible for 29% of outbreaks of bacterial agents and 6% of viral outbreaks. These authors noted that contact with animals was also a potential transmission route for enteric bacterial disease outbreaks (11%).

Several risk factors unique to child-care settings can contribute to pathogen transmission. For example, the presence of diapered children, which by virtue of close and frequent proximity to fecal material, is the most common source of enteric pathogens. Of particular concern is the potential for spreading pathogens via providers' hands and clothing, through common use of diaper-changing tables and leakage from diapers (Sullivan et al., 1984). Petersen et al. (1986) showed that bacteria of fecal origin were frequently present on the hands of children in diapers and staff members who are changing diapers, as well as on the diaper-changing area. However, Pickering (1986) stated that the spread of enteropathogens from infected, non-toilet trained children without diarrhea is uncertain. Additionally, there is the potential for fecal contamination of fomites when children share

toys, eating utensils, and blankets. Another source of contamination is allowing diapered children to crawl on carpeted surfaces, which can become contaminated and subsequently are never properly disinfected. Finally, the common mouthing behavior of young children between 0-6 years old (Tulve and Suggs, 2002) can increase the likelihood of enteric pathogen transmission in child-care settings.

Contaminated food is also of concern. Storage, preparation, and service of foods in child-care settings may be left to relatively unskilled employees who have inadequate training in hygiene, sanitation, and safe food handling practices, thus increasing opportunities for foods to become contaminated. A further concern is the fact that young children (under four years of age), with their immature immune systems, are more susceptible to many common foodborne pathogens (Buzby et al., 2001; Enke et al., 2007). For example, feeding children with powdered infant formula with contaminated water or bottles has resulted in cases of *Enterobacter sakazaki* which could be introduced during the manufacturing process (Drudy et al., 2006). Another concern is that child-care workers who diaper infants and assist children with toileting might handle food without following proper hygiene and sanitation practices (Sullivan et al., 1984). An additional and perhaps emerging transmission route for GI illness in young children is contact with farm and domestic animals and their fecal matter, which has been shown to increase the risk for enteric diseases (CDC, 2001). During year 2004-2005, three outbreaks of *E. coli* O157:H7, with 173 cases in total, were reportedly due to children visiting a petting zoo at agricultural fairs in North Carolina, Florida, and Arizona (CDC, 2005). A nursery school visit to a dairy farm resulted in twenty children and three adult helpers becoming infected with *C. jejuni* (Evans et al., 1996).

Environmental Contamination

It is important to understand the likely means by which pathogens are transmitted in child-care environments. One of those is environmental contamination when, for instance, a child with diarrhea touches a toy or surface with fecally-contaminated hands. If another child then touches that same surface, her/she may become ill after placing his/her hands in the mouth (American Academy of Pediatrics. 2009). This is particularly relevant to infant and toddler rooms, since these younger children are more likely to put their fingers in their mouths. Since 1983, twelve studies have been conducted to determine the microbial load on various surfaces in child-care facilities (Table 1.4). Ekanem et al. (1983) recovered fecal coliforms from 32% (42/131) of hand samples and 36% (23/64) of environmental samples taken from five child-care centers. Similarly, Van et al. (1991) isolated fecal coliforms from 46% of toy samples (73/159) and 17% of hand samples (131/771) from six child-care centers. In both studies, samples were collected after a reported GI illness outbreak. Under non-outbreak circumstances, Weniger et al. (1983) found far fewer positive samples; 4.3% (17/398 samples) of their environmental samples were positive for fecal coliforms.

In a study to determine risk factors for GI illness, Laborde et al. (1993) identified that children in classrooms with fecal contamination had double the rate of diarrheal illness compared with children in classrooms without such contamination. These investigators concluded that such contamination, where fecal coliform contamination in moist areas (i.e. around faucets or sinks) is most prevalent, could be a significant predictor for risk of diarrhea in children attending child care centers. In a more recent study, Cosby et al. (2008) evaluated the microbial load [aerobic plate counts (APCs) and coliform counts] of samples collected

from one food serving surface, two food preparation surfaces, and one diaper-changing surface at six child-care centers in Knoxville, TN. These investigators reported significant differences in microbiological counts between centers. Coliform counts ranged from 0.15 to 1.41 CFU/50 cm^2 and the highest mean coliform count was observed in food preparation areas (0.81 log CFU/50 cm^2), followed by food serving areas (0.58 log CFU/50 cm^2), and diaper-changing areas (0.44 log CFU/50 cm^2). It was hypothesized that the low coliform counts in the diaper changing areas was most likely due to the frequent use of sanitizers on these surfaces.

A similar type of study was conducted by Staskel et al. (2007) to evaluate the hygienic conditions of foodservice surfaces in 36 Texas child-care centers. Swab samples were collected from food-contact surfaces, such as countertops, and non-food-contact surfaces, such as hand-washing sink handles. Evidence of bacterial contamination was observed in 41% (68/167) of swab samples analyzed using a combination of culture, biochemical, and molecular screening techniques. The most commonly contaminated surfaces were sink drains, hand-washing sink faucet handles, and garbage can lid handles. Further bacterial strain typing yielded 27 different species. *Enterobacter cloacae*, a non-pathogenic species that is usually of fecal origin, was the most prevalent. *Salmonella paratyphi* A was also found in association with a sink area, however the disease caused by this bacterium is mainly in less developed countries with poverty and poor hygiene and very rare in the U.S.

The introduction of new molecular techniques in the 1990s has made it much easier to detect the presence of environmental contamination with viruses. Boone and Gerba (2005)

tested 218 fomites from 14 child-care centers for evidence of influenza viral contamination, reporting influenza A viral RNA in over 50% of the samples by RT-PCR. Using a similar technique, Wilde et al. (1992) demonstrated that about 26% (15/57 samples) of the fomites or environmental samples obtained from two child-care centers associated with a diarrheal disease outbreak showed evidence of rotavirus RNA. Other methods have also been used to evaluate potential fecal or microbial contamination in domestic home settings. For instance, the presence of hemoglobin has been used as a marker for blood; amylase as an indicator for the presence of urine, saliva and sweat; and protein as an indicator of general hygiene. Bellamy et al. (1998) used these techniques on 1,513 environmental samples collected from 39 homes. Amylase, which may be the result of contamination by saliva and sweat, was found in 29.3% of samples, indicating sampled surfaces may not be adequately cleaned. Protein, which is used as an indicator of general hygiene, was found in 97.8% of samples, which indicated inadequate general hygiene on sampled surfaces. Clearly, inclusion of alternative chemical testing methods that could be indicators of filth or fecal contamination might increase the robustness of future studies of environmental contamination in child-care settings.

Studies intended to evaluate microbial load on surfaces have both advantages and disadvantages. Clearly, this is the most direct way in which to determine if dangerous microbes are present. However, non-spore-forming bacteria, and particularly the Gram negatives, tend to lack environmental persistence, especially under dry conditions. Hence, they are usually only detectable after a recent contamination event. Molecular-based methods, while exquisitely sensitive, fail to discriminate between infectious and non-

infectious pathogens. The relationship between the presence of the classic fecal indicator bacteria (fecal coliforms, *E. coli*) and enteric pathogens like *Salmonella* is tenuous at best though they could quantitatively characterize the hygienic level of the sampled environment. Finally, studies that find occasional evidence of opportunistic pathogens on surfaces (i.e., Staskel et al., 2007) must be interpreted with caution, as these are frequent and common environmental contaminants whose presence presents little in the way of public health risk. Clearly, there is a need to establish better ways to assess surface contamination and novel methods such as those used by Bellamy et al. (1998) may prove useful in the future.

Observational Studies

Observational studies provide evidence about real-world practices that could lead to pathogen transmission and subsequent exposure of young children in child-care settings. A summary of observational studies carried out in child-care settings is summarized in Table 1.5. Each is described in more detail below.

A cross-sectional study design was used by Chapman et al. (1995) to evaluate hygiene practices in group and family day-care homes in order to determine if these practices are associated with the frequency and types of illness reported in enrolled children. A total of 204 licensed family homes and 137 group day-care homes in Washtenaw County, Michigan, were surveyed by questionnaire. The investigators found that the absence of attention to hand hygiene and decontamination of sleeping mats, as well as sharing cloth towels, increased the risk of illness among the children in day-care homes.

Other observational studies were designed to assess illness management decision-making in the child-care environment. For example, Enke et al. (2007) mailed a

questionnaire to 118 child-care center directors in Texas and Iowa to identify safe food handling, and other practices that influence the training and decision making of child-care administrators, relative to reducing the risk of foodborne disease in children. The investigators found that food service training curricula was provided to less than one third of the staff employed by surveyed facilities, and there is no universal food safety standard for any of the facilities visited. Taylor et al. (2008) interviewed 40 child-care workers from regulated centers in Southern Ontario, Canada via five different focus groups. They reported that most child-care center staff relied on experience and judgment in addition to public health information, to assist decision-making in the management of enteric illness and outbreaks; this was done instead of referring to specific child-care center acts and regulations. While the authors believed that this demonstrated staff dedication and responsibility, they also recommended that additional circumstance-specific guidance and advice from public health officials would be useful in helping staff meet regulatory requirements.

In a similar type of study, Copeland et al. (2006) collected data by mailing questionnaires to 215 Maryland pediatricians, 223 parents, and 192 child-care providers from 22 child-care centers. They reported that 77% (166/215) of pediatricians and 29% (56/192) of child-care providers were not familiar with official illness exclusion guidelines issued by the American Academy of Pediatrics (AAP)/American Public Health Association (APHA). Child-care providers and parents correctly excluded 65%-98% of cases requiring temporary exclusion of children with symptoms of six common childcare-associated illness including

gastroenterititis , while pediatricians correctly excluded 31%-86% of cases requiring exclusion, suggesting that pediatricians may underexclude sick children from child care.

Observational study designs have also been used to assess hygiene interventions in the child-care environment. For example, Sandora et al. (2005) conducted a cluster randomized, controlled trial of 292 families with children who were enrolled in 26 child-care centers. Intervention families received a supply of hand sanitizer and biweekly hand-hygiene educational materials for five months; control families received only materials promoting good nutrition. Primary caregivers were phoned biweekly and asked about respiratory and GI illnesses in family members. The investigators found that the GI illness rate was significantly lower in intervention families compared with control families (incidence rate ratio [IRR]:0.41; 95% confidence interval [CI]:0.19–0.90). While a relatively large study, the author also noted that the participants in their study generally had high educational levels and incomes making it difficult to generalize the results for families of different cultural backgrounds or lower socioeconomic status.

Kotch et al. (1994) developed a multi-component curriculum on handwashing and diapering skills for staff and delivered to 24 large child-care centers. Information about diarrheal and respiratory symptoms was obtained by telephone interview; the investigators also observed hygienic practices in the classroom. Their intervention resulted in a moderate reduction in the frequency of diarrheal disease (4.06 episodes/child-year for intervention facilities vs 4.95 episodes/child-year for control facilities). In a second study, the same group of investigators equipped 23 child-care centers in North Carolina with new diaper-changing, hand-washing, and food preparation equipment and compared to 23 control centers (Kotch et

al., 2007). Diarrheal illness prevalence was determined by phone calls to children's families. Diapering, hand-washing, and food preparation equipment that were specifically designed to reduce the spread of infectious agents significantly reduced diarrheal illness among the children; absenteeism among staff in out-of-home child-care centers due to illness was also decreased. However, the cost of purchase and installation, averaging $10,385 ($7,500 for the equipment and the rest for installation) per classroom, may be prohibitive for many child-care facilities.

While observational studies provide relevant, meaningful results in posing hypotheses on causal relationships, they alone can seldom establish clear causality, especially if not accompanied by parallel clinical or microbiological analyses (Gibson et al., 2002). Common deficiencies in such studies include failure to adequately control for potential biases and to provide sample sizes and diversity that allow for the production of widely generalizable results. Besides, due to the funding constraints, most of observational studies focus on a certain geographic area, or a demographic population, which limits generalizability of the results. Further, observational studies rarely include a microbiological component, with the exception of the early work of Laborde et al. (1993), who assessed the incidence of diarrheal disease in 221 children under the age of 3 years cared for in 24 child-care centers in North Carolina. That study used data on fecal coliform counts levels on the hands of children and staff, and on surfaces, to investigate a potential correlation to diarrheal illness. The investigators found an increased risk of diarrhea with increased evidence of fecal contamination on staff and children hands or sinks in child care centers. Clearly, an

integrated approach including both observational and microbiological analyses could maximize the utility of information produced in future studies.

Best Practices for Prevention and Control of Enteric Pathogen Transmission in Child-Care Settings

Measures to control transmission of diarrheal disease in child-care settings are well documented in the literature and in basic public health manuals like the American Academy of Pediatrics Red Book (2012) and American Public Health Association (APHA) Control of Communicable Diseases (2008). In general terms, measures used to prevent or contain an outbreak include vaccination; restricting infected children from attending a center (such as illness management); management of symptomatic or recuperating children; prophylactic treatment; center closure; environmental cleaning; improved hand-hygiene; and safe food handling. In most instances, preventing or controlling an outbreak requires the use of multiple control strategies simultaneously. For example, in 1995, practices thought effective in controlling an *E. coli* O157:H7 outbreak in Colorado included hygienic measures, such as increased hand-washing, cleaning and sanitization of toys and fomites, as well as cohorting of ill or convalescing children (Williams et al., 1997). Likewise, implementation of cleaning procedures and enhanced hand-washing were effective in controlling a 2000 shigellosis outbreak in Australia (Genobile et al., 2004).

Vaccination

At present, preventive vaccination is only an option for the control of Hepatitis A virus and rotavirus; vaccines are not available for other common enteric pathogens. Belmaker et al. (2007) documented the effect of Hepatitis A virus vaccination in the child

care environment. Specifically, universal toddler immunization against hepatitis A virus was implemented in 1999 in southern Israel, with no subsequent outbreaks of this disease reported in child care centers or schools in that region since 2000. Others have also shown that vaccination of children two to five years of age enrolled in licensed child-care centers has proven to be an effective preventive measure for control of hepatitis A virus transmission, even after recent exposure (Venczel et al., 2001; Victor et al., 2007). As a consequence, the American Academy of Pediatrics (2011a) recommends that, nationally, all children should receive their first dose of Hepatitis A virus vaccine between 12 and 23 months of age, although the vaccine can be given at older ages. The second dose should be given at least six months following the first dose. Rotavirus vaccination is also an effective control (Coffin et al., 2006) and approved by U.S. Food and Drug Administration (FDA) to be safely administered to children (Food and Drug Administration, 2010). This vaccine is delivered in three doses that are given by mouth at two, four, and six months of age (American Academy of Pediatrics, 2011b).

Illness Management

The most common symptom of GI illness is diarrhea followed by vomiting and fever. Infected patients usually shed pathogens in their stools preceding symptoms; they can also shed while symptomatic and even post-symptomatically. For example, children shed *E. coli* O157:H7 for a median of 29 days during one outbreak in Colorado (Williams et al., 1997). Vomitus can also be an important vehicle for norovirus transmission in childcare facilities, although its relative importance is poorly characterized (Contra Costa Health Services, 2012).

Managing GI illness is further complicated by the fact that for some diseases, not all infected persons become symptomatic. For instance, a community outbreak of hepatitis A infection was traced back to contact with children attending a particular child-care center, yet none of the children had evidence of overt illness (Sadetzki et al., 1999). Hellard et al (2000) analyzed 1091 fecal samples from asymptomatic individuals, finding 28 known pathogens with a total carriage rate of 2.6%. *Giardia* species were present in 1.6% or samples, *Salmonella* in 0.4% or samples, and *Campylobacter* in 0.1% or samples. The median age for children shedding these pathogens was 6.6 years. Notwithstanding the potential for subclinical infection, it should be clear that a child with symptoms of GI illness who continues to attend a child-care center may ultimately infect other children attending that center. Therefore, it is very important for facilities to develop an illness management policy that addresses exclusion and isolation of infected children, with or without symptoms (American Academy of Pediatrics, 2002).

Many public health experts have suggested that exclusion of ill children from child care be used as a means to reduce the transmission of disease to healthy children and to prevent the spread of infection in the community at-large. In a very cautious move, Pickering (1986) advocated for the exclusion of all non-toilet trained children experiencing loose stools, simply because of the difficulty in controlling disease spread under these circumstances. However, the decision to exclude is a complicated one that is fraught with opposing social pressures from working parents who need child care, and parents of well children who do not want their child exposed. As described above, exclusion of ill children from child care is costly for parents and employers (Kahan et al., 2005). In addition, there

may be a level of deception. For example, when a child is excluded from one child-care center, parents sometimes seek another center that is unaware of the child's health status (National Disease Surveillance Centre, 2003). In other instances, parents will even attempt to conceal that their child is ill (Williams et al., 1997). Exclusion of ill staff members is equally important, as illustrated by an outbreak associated with child-care center catering staff, who were responsible for spreading Norowalk-like virus causing gastroenteritis among 195 children attending 30 different centers in Sweden. This occurred despite the fact that the staff members never came in direct contact with the children (Gotz et al., 2002). Unfortunately, there are few published studies that quantify the actual reduction in secondary cases that can be achieved by excluding sick children, providers, and staff. Studies on the exclusion of sick children primarily focus on when a child must be excluded because the risk of transmission is extremely high (Aronson and Osterholm, 1986). For example, the American Academy of Pediatrics Red Book (28th edition) recommends exclusion for children infected with severe enteric diseases caused by Shiga toxin-producing *E. coli, Shigella*, and *Salmonella enterica* serotype Typhi. In these cases, children should remain excluded until multiple stool cultures test negative for the pathogen. However, the inclusion of asymptomatic children can increase the risk of disease transmission because asymptomatic carriers frequently shed pathogens in high numbers yet are indistinguishable from healthy children (Thompson, 1994; Gardner and Hill, 2001).

According to some public health experts, most children with a mild enteric illness should not be excluded from child care unless the child is unable to participate comfortably in group activities; the illness requires more care than the center can provide; or the child has

symptoms suggesting a more serious illness that requires medical attention (Kahan et al., 2005). Some have even suggested that, as a general recommendation, children with potentially contagious GI illness should be excluded from child care only if they cannot be cohorted within the same facility under the care of trained staff (Aronson and Osterholm, 1986; Copeland et al., 2006). The decision as to whether a child is actually admitted into formal child care ultimately belongs to the child-care provider and is usually made on a case-by-case basis (Brady, 2005).

Food handlers are also subject to exclusion from food preparation according to the U.S. Food Code (Food and Drug Administration Food Code, 2009), specifically when one is: (1) symptomatic with vomiting or diarrhea; (2) jaundiced or diagnosed with hepatitis A infection; (3) diagnosed with or reported previous infection due to *Salmonella*; (4) diagnosed with an asymptomatic infection from norovirus; (5) diagnosed with *Shigella* infection, even if asymptomatic; (6) diagnosed with enterohemorrhagic *E. coli*, even if asymptomatic; (7) symptomatic with sore throat with fever; or (8) symptomatic with an uncovered infected wound or pustular boil, or exposed to foodborne pathogen and works for food preparation. These criteria should also be applied for exclusion of food preparation staff at child care facilities.

Cohorting

"Cohorting," or separating convalescing children from healthy children after the former have spent the acute phase of the illness under care outside the center, is a practical approach for the child who still may be shedding the pathogen but feels well and is active (Ang, 2000; Drees et al., 2004; Ferson et al., 1997; Gouveia et al., 1998; Williams et al.,

1997). Cohorting can be effectively implemented by providing separate play areas, restrooms, and dining areas for convalescing and healthy children (Drees et al., 2004). This can be difficult for many child-care centers, as adequate facilities and staff would be necessary to implement effective cohorting (Lee et al., 2008). There is, however, precedent for cohorting, as illustrated after a 2002 shigellosis outbreak that occurred in Delaware. A total of 506 culture-confirmed cases (median age four years) were reported, 40% (200/506) of which were child care related (CDC, 2004). As a result, the Health Department of Delaware excluded children with diarrhea from child care. Diapered children were allowed to return after completing antibiotic treatment, and non-diapered children were allowed to return to facilities after 48 hours of antibiotic treatment. Brown et al (2011) also reported that cohorting is an effective control measure used in an outbreak (45 cases) caused by *E. coli* O26:H11 at a child care center in Colorado. By taking this approach, the outbreak was managed without having to close the facility.

Prophylactic Antibiotic

Prophylactic administration of antibiotics may sometimes be used to control further transmission of certain infectious diseases. Examples of this strategy include control of a British giardiasis outbreak in 1998 by treating healthy children with metronidazole (Ang, 2000). The prophylactic use of antibiotics must be approached cautiously, however, given recent concerns about emerging antibiotic resistance in pathogenic bacteria. For example, the *Shigella* strain isolated from a Portuguese child-care setting outbreak was resistant to tetracycline and co-trimoxazole, which is a mixture of trimethoprim and sulfamethoxazole) (Suspiro et al., 1996).

Center Closure

Overall, closing a child-care center is usually done only when there are serious or uncontrollable outbreaks or incidence or disease, (i.e., death, serious illness, or evidence of a reportable infectious disease), and not for relatively mild gastroenteritis (American Academy of Pediatrics, 2011c). For example, a 2002 outbreak of *E. coli* O157:H7 in Alberta, Canada, ended only after the child-care center voluntarily closed after eight children became symptomatic (Galanis et al., 2003). As described above, child-care center closure has significant economic ramifications to both the center and children's parents because it may disrupt the facilities normal operations of serving the children and their family (Pickering, 1986).

Environmental Sanitation

Regular cleaning and disinfection of environmental surfaces is important for prevention of enteric disease transmission (Pickering, 1986; Brady, 2005). For example, Keswick et al. (1983) detected evidence of rotavirus contamination in a child-care center in the U.S. by collecting and testing environmental swabs taken from the hands of teachers and various surfaces, including toys, phones, toilet handles, sinks, and water fountains. Of 25 samples collected, 16% (4/25) were positive for rotavirus. As well, Krilov et al. (1996) reported that disinfecting environmental surfaces or objects (like floors, toilet areas, taps and toys) in child-care environments can reduce the incidence of enteric viral disease from 0.70 illnesses/child-month to 0.53 illnesses/child-month.

Relative to older children, mouthing children are probably at a higher risk for enteric disease transmission due to their potential exposure to objects or surfaces contaminated by

pathogens. For this reason surface disinfection is crucial to reducing transmission. Toys, for example, are suggested to be cleaned three times per week with paper towels or clean cloth towels, followed by the application of disinfectant spray and air drying (Aronson and Osterholm, 1986; Krilov et al., 1996; Pickering, 1986). If this is not possible, toys should be removed from circulation (Krilov et al., 1996). The diaper-changing area is another important source of enteric pathogens. Child-care providers who change diapers should have a designated changing area per infant or toddler group with a surface suitable for sanitization after each change. The facility should not permit shared use of diaper-changing tables and sinks by more than one group (American Academy of Pediatrics, 2011d; Morrow et al., 1991). Aronson and Shope (2009) have provided recommended guidelines for cleaning diaper-changing areas, consisting of the following sequential steps:(1) appropriate disposal of the paper liner used on diaper changing surfaces (i.e. disposal in a plastic lined, covered, hands-free receptacle); (2) cleaning the surface of any visible soil using detergent and water, followed by a water rinse; (3) spray application of a sanitizing bleach solution over the entire changing surface with a contact time of at least two minutes; and (4) drying the surface by air or wiping using a disposable paper towel. Forty-two (42) states require child-care facilities to sanitize diaper changing areas after each use (NCCITAC/NARA, 2010; Table 1.6).

Hand Hygiene

Contaminated hands can be an important source of enteric pathogens, contributing substantially to their transmission in child-care environment (Miller et al., 2012). For example, both Goldmann. (2000) and Hall et al. (1981) noted that touching infants infected with respiratory syncytial virus (RSV) or surrounding fomites was a risk factor for

developing RSV infection in nurses. Hence, careful attention to hand-hygiene is an important infection control strategy. This is detailed further in a review article by Barker et al. (2001), who cited over 15 research studies that demonstrated a decrease in viral contamination and infection in the child-care environment when hand washing was used regularly as an intervention. Indeed, failure to implement proper hand-hygiene practices is probably the single most common reason for GI illnesses, and contributes to outbreaks of diarrhea among children, providers, and teachers in child-care settings (American Academy of Pediatrics, 2011e; Morrow et al., 1991). Appropriate hand hygiene is essential to preventing infection and controlling transmission for foodborne illnesses caused by *Cryptosporidium*, *E. coli* O157:H7, rotavirus, *Giardia lamblia*, Hepatitis A virus, *Shigella*, and norovirus (Heyman, 2004).

Environmental studies in child-care centers have shown that the hands of children and care providers are frequently contaminated with fecal coliforms (Van et al., 1990). However, Larson et al. (1986) expressed concerns that skin damage to the hands due to washing could change native microflora and ultimately increase bacterial antibiotic-resistance. The American Academy of Pediatrics (2011e) recommends that child-care staff perform recommended hand-hygiene practices throughout the day in association with the following activities: (1) upon arrival for the day, after breaks, or when moving from one child-care group to another; (2) before and after (a) preparing food or beverages, (b) eating, handling food, or feeding a child, (c) giving medication or applying a medical ointment or cream in which a break in the skin (sores, cuts, or scrapes) may be encountered, (d) playing in water (including swimming) that is used by more than one person, and (e) diapering; (3) after (a)

using the toilet or helping a child use a toilet, (b) handling bodily fluid (mucus, blood, vomit) from sneezing, wiping and blowing noses, from mouths, or from sores, (c) handling animals or cleaning up animal waste, (d) playing in sand, on wooden play sets, and outdoors, and (e) cleaning or handling the garbage.

The CDC recommends that the best way to decontaminate hands is by washing them for 10-15 seconds under warm water using plain or antimicrobial soap (CDC, 2002). Studies have also demonstrated that using an alcohol-based hand sanitizer after washing hands with soap and water is effective at reducing illness transmission in the home, in child-care centers, and in health care settings (Boyce et al., 2002; Sandora, et al., 2005). However, because hand sanitizer products might be toxic if ingested from the residue left on hands after rinsing, it is important for the care givers to monitor children carefully (American Academy of Pediatrics, 2011e). Further, alcohol-based hand sanitizers are not effective against some important enteric pathogens, particularly human noroviruses. Hand sanitizers should be viewed as an important secondary control, but only after hands have first been thoroughly cleaned using soap and water.

Seventeen (17) states have regulatory requirements about the location and number of hand-washing facilities available to staff in child-care facilities (Table 1.7). The District of Columbia and 46 states, including the District of Columbia, (CA, CO, HI, ID, LA are excluded) have regulatory requirements that specify times when hand washing is required for center staff: after diapering children (45 states); before and after preparing, serving, and eating food (41 states); after toileting (39 states); after toileting children (33 states); after handling, feeding, and cleaning up after animals (22 states); and after attending to ill children

(9 states; NARA, 2010). Forty-four (44) states also have hand-washing requirements for children in child-care centers, and of those states only one, Colorado, does not specify when children must wash their hands (NCCITAC/NARA, 2010). In short, regulatory requirement for hand washing in child care centers are not standardized and vary widely among states.

Numerous studies support the efficacy of training children and staff on the best methods to approach hand washing in the child-care environment (Ponka et al., 2004; Roberts et al., 2000; Uhari et al., 1999). For example, a randomized, controlled trial conducted by Roberts et al. (2000) in which child-care staff were trained about infectious disease transmission and the importance of hand washing revealed that the incidence of diarrhea was reduced to 1.9 episodes per child-year in the intervention centers compared to 2.7 in the control centers (311 children observed during one year from 23 day-care centers in total).

Proper hand-washing equipment specifically designed for children is another important issue. According to the American Academy of Pediatrics (2011d), a hand-washing sink should be accessible without barriers (such as doors) to each child-care area. In areas for infants, toddlers, and preschoolers, the sink should be located so that the providers and teachers may visually supervise the group of children while carrying out routine hand washing or having children wash their own hands. Sinks should be placed at the appropriate height or be equipped with a stable step platform to make the sink available to children. If a platform is used, it should have slip-proof steps and a platform surface. Also, each sink should be equipped so that the user has access to:(1) water, at a temperature of at least 60°F and no hotter than 120°F; (2) a foot-pedal operated, electric-eye operated, open, self-closing,

slow-closing, or metering faucet that provides a flow of water for at least thirty seconds without the need to reactivate the faucet; (3) a supply of hand-cleansing non-antibacterial, unscented liquid soap; and (4) disposable single-use cloth, or paper towels, or a heated-air hand-drying device with heat guards to prevent contact with surfaces that get hotter than 120°F. Guidelines further state that hand-washing sinks for children should not be used for rinsing soiled clothing, for cleaning equipment that is used for toileting, and/or for the disposal of any waste water used in cleaning the facility. The results from Kotch et al (2007) suggested that childcare facilities installing handwashing equipments with automatic faucet had lower rate of diarrhea in children, however high cost of installment may set barrier of the equipment to become common in the childcare facilities.

Safe Food Handling

Fifty-one (51) outbreaks in child-care facilities have been linked to contaminated food, mainly from fruits, vegetables, meat, poultry and dairy product (Table 1.3). For example, investigation of the community-wide shigellosis outbreaks in Kentucky revealed that day-care centers, which have had a food handler who also was responsible for diapering children, are more likely to have enteric disease outbreaks than those which have different personnel for food preparation and diaper changing (Mohle-Boetani et al., 1995). Fifty-six (56) laboratory-confirmed cases of cryptosporidiosis were identified from two child-care centers in Missouri, where pool water probably served as the vehicle for disease transmission. The highest risk for infection was associated with eating at the pool (adjusted odds ratio, 7.26; 95% confidence interval, 2.57-20.48; Turabelidze et al., 2007). Observational studies of food handlers in restaurants support the frequent occurrence of

unsafe food preparation practices (Clayton et al., 2004; Howes et al., 1996; Manning et al., 1993); less is known if these same practices occur in childcare settings. However, a unique risk to this setting is the presence of diapered children, and it is recommended that diapering staff should not also handle food. The risk associated with such a practice was illustrated by Sullivan et al. (1984), who reported that licensed child-care facilities in Texas where diapering and food handling were done by the same caregiver, had marginally higher rates ($0.05<p<0.10$) of child diarrheal disease.

Not only is food handling an important consideration, but so is the source of the food. Ten cases of salmonellosis associated with *S. enterica* serovar Typhimurium PT135a occurred in association with a child-care facility in Brisbane, Australia, following consumption of eggs purchased from a supplier without a quality assurance program (McCall et al., 2003). Finally, special guidelines that are recommended for the preparation of baby food, breast milk, or bottles for infant food should be carefully followed in day-care settings (Fight Bacteria, 2012). A study conducted by Day et al. (2011) found that *Salmonella* Typhi and *Shigella dysenteriae* can remain viable for prolonged period of time in powdered formula, which provided proof to the necessity of carefully following guidelines for preparing baby formula.

Training

Training requirements for child-care providers vary widely among states and across facility types (Table 1.7). Not surprisingly, child-care centers are subject to more training requirements than are child-care homes. All but one state require child-care center workers to meet specific pre-service qualifications, typically defined as training, education, and

experience prior to employment. In addition, ongoing training (usually 12-15 contact hours per year) is mandatory for center directors, teachers, and aides in most (46) states. Forty-four (44) states require small family day-care home providers to complete ongoing training, at least four hours every one to two years. Providers working in large/group family day-care homes are also required to complete ongoing training in 36 states (National Association for Regulatory Administration, 2010) (Table 1.7).

The focus of training is detailed in most state regulations. For example, all states except Idaho and Missouri require child-care center staff to complete health and safety training. While most require first aid (47 states) and CPR training (46 states), only half of states require child-care center staff to complete training on prevention of communicable diseases. Few states require training on communicable disease prevention for providers working in small and large/group family day-care homes (National Association for Regulatory Administration, 2010; Table 1.7).

Only nine states (CA, CT, GA, IL, MD, OH, TN, TX, WI) require child-care center staff to be trained on how to care for ill children (Table 1.7) even though most states (47) have requirements that describe how to cohort mildly ill children. This is important because 18 states allow children to attend a child-care center when they are mildly ill. All states that allow mildly ill children to be present in a child-care center have regulations describing supervision of ill children, the types of activities centers should make available to ill children, and facilities where ill children should be cared for. Three states (AZ, CO and GA) require providers working in large/group family day-care homes to receive training about the care of ill children, but no state requires this type of training for providers working in small family

day-care homes. Similar to child-care centers, states have requirements that address the care of mildly ill children in small and large family day-care homes (35 and 29 states, respectively) (National Association for Regulatory Administration, 2010). Only twelve states allow children who are sick to be in a small family day-care home and nine states allow mildly ill children to be in a large/group family day-care home.

The lack of required training on how to prevent communicable diseases is a serious concern that must be addressed if incidents of illness are to decrease in child-care settings. When child-care staff are knowledgeable in general hygiene and sanitation practices, programs are more likely to be healthy and safe for children (Alkon et al., 2009). In fact, compliance with continuous staff education was reported to be the most significant predictor for compliance with state child-care health and safety regulations in Connecticut (Crowley and Rosenthal, 2009). Specifically, training should also be directed more towards the child-care home workers because they are generally less educated about the importance and methods of preventing communicable diseases (Slack-Smith et al., 2005).

An online search for training materials targeting child-care providers yielded hundreds of resources. However, the effectiveness of the training materials is relatively unknown. To date, only three studies have been published about specific food safety knowledge and practices of child-care workers. The findings from all three studies reinforce the need for training of child-care workers about hygiene, sanitation, and food handling. Specifically, Albrecht et al. (1992) observed that child-care staff lacked general knowledge and commitment to quality foodservice principles, which in turn contributed to foodborne illness in young children in child-care facilities. Enke et al., (2007) and Sangster et al.,

(2004) also identified a general lack of compliance with recommended food handling practices by workers in child-care settings. Nonetheless, training interventions that address improved sanitation, hand-hygiene, and food preparation practices have been shown to significantly reduce the frequency of diarrheal disease among children and staff in child-care facilities (Kotch et al., 2007). Studies have shown that conducting proper hand-washing practices before or after a potentially contamination event reduces the extent of infection in child-care settings, and decreases risk of transmission (Brady, 2005; Thompson, 1994). To promote infection control, Soto et al. (1994) introduced a hand-washing technique among educators and children attending 40 day-care centers in Canada. Efficacy of hand washing was assessed using a topographic scale of the hands and a fluorescent marker which is removed by adequate hand washing. The trained personnel showed a continuous improvement of hand-washing "scores" over time (1989, 80.3%, 1990, 82.4%, 1991, 90.5%). This enhancement was most noticeable for nails (1989, 52.6%, 1990, 65.6%, 1991, 80.2%) and wrists (1989. 55.4%, 1990, 72.9%, 1991, 85.4%). Among children, the mean score was 76% (obtained in an average of 37 seconds of washing). Lower diarrhea rates were also associated with the best-scoring groups.

One factor that has not been addressed, which could have a negative impact on the effectiveness of a training program, is the competency of the educator who is delivering the information. Published literature from consumer behavior, social psychology, and related disciplines suggests that a highly credible source is more likely to lead to increased behavioral compliance than a low credible source (Sidney and Shuv-Ami, 1986; Maddux and Rogers, 1980). For example, Jones et al. (2003) found that participants receiving a positive

communication from a credible source reported more positive exercise intentions and behaviors than did participants who received information from a non-credible source. Other studies have also shown that the degree of perceived credibility of the source influenced a recipient's intention to use suggestions made by that source (Bannister, 1986). Therefore, training initiatives must put more emphasis on helping the educator to become credible and improve competency. This can be achieved by developing training interventions to improve educators' scientific understanding of risk factors and their controls as related to the child-care environment. An educator who can explain the science behind the practices will be more credible.

Communication between child-care staff and parents is another form of education that is also essential. One example was reported by Abraham et al., (1997), in association with a 1996 child-care center closure in Ontario, Canada, due to an outbreak of *E. coli* O157:NM. The staff informed the parents about the outbreak by letter, and the parents were requested to monitor their children for symptoms of enteric disease. Facility staff also conducted an informational night to advise staff and parents on the importance of hand washing. Another important area for parental education is the proper handling and labeling of foods that are sent with the child to the care facility, such as breast milk, snacks, and lunches. Thirty-five (35) states allow parents and guardians to provide food for their children while attending a child-care center. Twenty-two (22) states allow this practice in small family day-care homes, while 27allow it in large/group family homes (National Association for Regulatory Administration, 2010).

Regulations

Across the U.S there are 329,882 licensed child-care facilities of which 32.49% are child-care centers and approximately 60% are family day-care homes (NCCITAC/NARA, 2010).Children presumably benefit from licensing with respect to the spread of infectious diseases, prevention of fire, and other building safety hazards, as well as injury and developmental impairment that could potentially result from the irresponsible behaviors of untrained and unregulated workers (Children & Youth Partnership for Dare County, 2011).

Licensure provides an opportunity for a third-party, typically the local regulatory agency, to assess practices through the process of inspection (Rhode Island Department of Children, Youth and Families, 2012). Licensure is defined as the granting of permission to operate a child-care facility by a local or state regulatory authority, and to receive a license, a facility must meet a set of baseline standards. Only one state, Idaho, does not license child-care facilities at the state level; Idahoan licensure occurs at the local level. Child-care facilities in Anchorage, Alaska, New York City, and select counties in Florida are also licensed at the local level. Seven states (AZ, ID, LA, NJ, OH, SD, VA) do not license small family day-care homes. Eleven states and the District of Columbia (AR, DC, ID, KY, LA, MD, ME, NC, NJ, VT, WA, WI) do not license large/group family day-care homes (NARA, 2010).

Child care facility inspection

Supervision and monitoring of child-care facilities are critical to facilitate continued compliance. The position statement of the National Association for the Education of Young Children says, *"Effective enforcement requires periodic on-site inspections on both an announced and unannounced basis with meaningful sanctions for noncompliance."* NAEYC

also recommends that all centers and large and small family child-care homes receive at least one site visit per year. Unannounced inspections have been shown to be especially effective when targeted to providers with a history of low compliance (NAEYC, 1997).

Similar to state-level licensing, all states except Idaho require inspections of child-care centers prior to issuing a license to ensure compliance with regulations. In 34 states the visits are announced, giving the facilities time to prepare (Table1.7). Inspections also occur at times of license renewal and as part of routine compliance during the licensing period. The frequency of inspections in child-care centers varies widely, from more than three times per year in Nevada and Tennessee, to less than once every three years in California. Twenty-six (26) states require child-care centers to be inspected once per year. Inspection frequency decreases in small and large family day-care homes, with some states not requiring any inspection. In addition to inspections to determine compliance with regulations, 40 states require environmental health inspections in child-care centers. Only 12 states require environmental health inspections for small family day-care homes, and 17 states require inspections for large/group family day-care homes (National Association for Regulatory Administration, 2010).

Care of Children

Almost all states have regulatory requirements that detail the daily activities that a child-care center must provide each day to meet a child's developmental needs. Toileting and hand washing are included in this category, with only 16 states requiring this activity in child-care centers (Table 1.7). Five states (AK, IL, MO, RI, WV) require this in small family

day-care homes and eight states (AK, CT, GA, IL, MO, OR, RI, WV) in large/group family day-care homes (NCCITAC/NARA, 2010).

Food service in child care

Twenty (20) states require child-care foodservice operations to meet the FDA Food Code requirements. A comprehensive set of standards is available from the National Resource Center for Health and Safety in Child Care and Early Education. Specifically, *Care for our Children: National Health and Safety Performance Standards* (American Academy of Pediatrics, 2011f) lists food safety standards for (1) the preparation, feeding, and storage of human milk; (2) the preparation, feeding, and storage of infant formula; (3) the cleaning and sanitizing of equipment used for bottle feeding; (4) the cleaning and sanitizing of tableware and eating utensils; (5) the maximum numbers of children fed simultaneously by one single adult; and (6) safe treatment and storage of leftover foods. Additionally, 48 states have requirements about feeding infants in child-care centers; thirty-six (36) states have these requirements in small day care homes—thirty-three (33) states for large/group family day-care homes (NCCITAC/NARA, 2010; Table 1.7).

Conclusions

Formalized childcare is an indispensable part of American society, as well as in many other of the world's societies. With its many advantages, however, are accompanied increased enteric disease risks, which inflict significant financial and public health burden. While strides have been made on how enteric diseases are transmitted and should be treated in the childcare setting, much remains unknown. In this paper, we have discussed, in detail, the evidence supporting enteric disease transmission in childcare settings. We have also

outlined the various control measures, including discussion of their efficacy and potential barriers to their implementation. Finally, the paper discusses licensure and inspection practices throughout the U.S., which vary widely by state. This is all a prelude to our project, the purposes of which was to identify risk factors for foodborne infections in the child care environment and to develop more effective and targeted interventions for foodborne illness prevention. The following four specific aims were identified:

1. To develop a more complete characterization of food handling, hygiene, and sanitation practices that contribute to foodborne illness by conducting observations of child care workers at child care facilities in NC and SC.

2. To collect and analyze microbiological samples from child care workers' hands and surfaces at these same child care facilities in NC and SC.

3. To identify foodborne illness risk factors and effective control strategies using the findings from the observations and microbiological testing results.

4. To develop, deliver, evaluate, and disseminate training interventions targeting food safety educators based on identified risk factors and control strategies to increase their competency in delivering educational messages to child care workers.

In this dissertation, the data and analysis associated with Specific Aims 1, 2 and 3 are described.

Table 1.1 Pathogens and Morbidity Data Linked to 75 Diarrheal Outbreaks Associated with Child-Care Centers (Lee and Greig, 2008).

Pathogen	Number Of Outbreaks (%)	Number Of Illnesses	Confirmed Cases	Number Hospitalized
Bacteria				
Escherichia coli	20 (26.7)	299	227	64
Salmonella spp.	6 (8.0)	176	125	4
Shigella spp.	4 (5.3)	139	22	5
Yersinia enterrocolitica 0:8	1 (1.3)	42	16	0
Campylobacter jejuni	1 (1.3)	20	15	0
Staphylococcus aureus	1 (1.3)	19	1	0
Bacillus cereus	1 (1.3)	6	0	0
Unknown	1 (1.3)	15	0	2
Subtotal	**35 (46.7)**	**716**	**406**	**75**
Viruses				
Hepatitis A virus (HAV)	8 (10.7)	172	126	2
Norovirus	8 (10.7)	213	33	0
Rotavirus	7(9.3)	268	168	8
Astrovirus	3(4.0)	116	38	0
Calicivirus	2(2.7)	35	4	0
Adenovirus	2(2.7)	30	3	0
Sapovirus	2(2.7)	30	26	0
Echovirus	1(1.3)	39	39	13
Multiple organisms	2(2.7)	103	44	6
Subtotal	**35 (46.7)**	**1006**	**481**	**29**
Parasites				
Cryptospordium	2(2.7)	47	40	0
Giardia spp	2(2.7)	22	15	0
Blastocystis	1(1.3)	15	4	0
Subtotal	**5(6.7)**	**84**	**59**	**0**
Total	**75 (100)**	**1806**	**946**	**104**

Table 1.2 Summary of Published Gastrointestinal Illness Outbreaks in U.S. Child-Care Centers

Etiological Agent	Cases	Transmission Route	Control Measures	Reference
Cryptosporidium	18	Fecal-oral	Not reported	Bernhard (2010)
E.coli	8	Person-to-person	Closure	Curran (2010)
E.coli O157:H7	4 (1 death)	Person-to-person	Closure	Mallove (2010)
E.coli O157:H7	14	Not reported	Separated children with symptoms	Nieto (2010)
E.coli O157:H7	29	Not reported	Closure of center until none show symptoms	Falkenstein (2010)
Norovirus	18	Fecal-oral	Improved hand washing and sanitation; exclude ill children and staff	Ghosh et al. (2010)
E.coli O157:H7	22	Not reported	Closure and strict cleaning	Whitney et al. (2009)
E.coli O157:H7	11	Fecal-oral	Not reported	Parker et al. (2009)
Astrovirus	26	Not reported	Exclusion of symptomatic children; mandated testing of all symptomatic staff ; testing of symptomatic children; and temporary closing of the facility	Finkbeiner et al. (2009)
Shigella	9	Fecal-oral	Hand washing, food preparation hygiene, send sick staff home, separate food and diapering areas	Ghosh et al. (2009)

Table 1.2 Continued

Etiological Agent	**Cases**	**Transmission Route**	**Control Measures**	**Reference**
Rotavirus group A (17%) Norovirus (10%) Sapovirus (7%) Multiple viruses (10%)	29 (outbreaks, case was not reported)	Fecal-oral	Not reported	Lyman et al. (2009)
Norovirus	Est. 7880	Fecal-oral	Not reported	Doyle et al. (2009)
Norovirus	41	Person-to-person	Closure and cleaning	Doyle et al. (2008)
E.coli O157:H7	6	Not reported	Not reported	Fennelly et al. (2007)
E.coli O157:H7	6	Person-to-person	Not reported	Turabelidze et al. (2007)
E. coli O157:H7	11	Not reported	Staff education, hand washing, and cohorting or exclusion of attendees with diarrhea	Fennelly et al. (2007)
Cryptosporidium	56	Recreational pool water	Not reported	Turabelidze et al. (2007)[a]

[a] Also occurred in the community.

Table 1.3 Summary of CDC Data on Foodborne Illness Outbreaks in Child-Care Centers (1998-2008)

	Etiological Agent			
	Bacteria	**Viruses**	**Unknown**	**Total**
Number of Outbreaks	28	9	14	**51**
Total Ill	1286	337	324	1947
Total Hospitalized	74	1	2	77
Total Deaths	0	0	0	0
Implicated Foods	Brownies Cantaloupe Chicken and rice Chicken lo mein Green beans Ground beef Hard boiled eggs Honeydew melon Strawberries Turkey Watermelon	Lettuce-based Salads	Buffalo wings Chicken Coleslaw Macaroni and cheese Pizza Pork, BBQ Salads Turkey sandwich	

Table 1.4 Summary of Microbiological Surveys Conducted in Child-Care Facilities

Authors	Microbiology Test Methods	Study Period	# of Facilities	# and Type of Samples	Findings
Cosby et al. (2008)	APC *E. coli* Coliforms	8 months, sampling twice per month	6	288 from three food-contact surfaces and one non-food-contact surface	No correlation between contamination and illness in facilities
Staskel et al. (2007)	Typing of organisms isolated from surfaces; 27 bacteria isolated, mostly opportunistic Non-opportunistic *S. paratyphi* A and *Klebsiella pneumonia* also isolated	NS	36	167 from faucets, sinks, trash can lids, cutting boards.	Most common areas of bacterial contamination were sink drain area of dishwashing sink, hand-washing sink faucet handles handle of garbage can lid, and cutting boards.
Boone and Gerba (2005)	Influenza A virus	2.5 years	14	218 (fomites from kitchens, play areas, living areas, and bathrooms).	Influenza virus detected on 23% samples in fall and 53% samples in spring. No virus detected on home samples during summer; 59% of samples in March.
Bellamy et al. (1998)	Enteroviral RNA Hemoglobin, amylase, saliva, sweat, protein	2 five-week sessions	39 homes (17 visited on 2 times)	448 (toilets, washbasins, baths, telephone, babies, and kitchen)	Enteroviral RNA detected in 3/448 samples. Samples were taken from a tap handle, telephone hand piece and toilet bowl.

Table 1.4 Continued

Authors	Microbiology Test Methods	Study Period	# of Facilities	# and Type of Samples	Findings
Laborde et al., (1993)	Fecal coliforms	7 months	24	Number not specified (hands, faucets, sinks, diapering tables, floors, and toys)	Significant predictors of diarrheal risk associated with hand contamination (p=0.003) and number of contaminated moist sites (hands, faucets and sinks) (p=0.006).
Butz et al. (1993)	Rotavirus APC Total fungal counts	6 months but in different seasons	2	96 (high-touch fomites, water, and play tables)	18/96 samples positive for rotavirus RNA. Common contamination sites were phone receivers, drinking fountains, water-play tables, and toilet handles.
Wilde et al. (1992)	Rotavirus	3 months	4 (2 with outbreaks, 2 without)	122 (floors, diaper change areas, and toys)	In two centers with reported rotavirus outbreaks, 7/18 toys had detectable rotavirus RNA as did 8/39 swabs from environmental surfaces. In centers without outbreaks, 1/21 toy ball and 1/44 environmental surfaces swabs had detectable rotavirus (P=0.0001).
Van, R et al. (1991)	Fecal coliforms	13 weeks	6	2953 (inanimate objects and hands)	Fecal coliform contamination common and greater (P<0.05) for objects, toy balls, and hands of children in toddler rooms compared with infant rooms. Occurrence of diarrhea was significantly associated with increased hand contamination (P=0.001).

Table 1.4 Continued

Authors	**Microbiology Test Methods**	**Study Period**	**# of Facilities**	**# and Type of Samples**	**Findings**
Ekanem et al. (1983)	Fecal coliforms Giardia *Shigella* *S.* Typhimurium	9 months	5	131 hand samples, 64 classroom surface samples.	During outbreaks of diarrhea, fecal coliforms were recovered with significantly greater frequency of hands (32%; $p<0.005$) and from classroom objects (36%; $p<0.005$).
Keswick et al. (1983)	Rotavirus	N/A	1	25 (environmental surfaces and teacher hands.	Rotavirus viable on contaminated surfaces long enough to be transmitted to susceptible children.
Weniger et al. (1983)	Fecal coliforms	1 month	2	398 from bathroom, diaper changing areas, doors, floors, kitchen areas, furniture, and toys.	Fecal coliforms found in 17/398 samples of building surfaces, furniture, and other objects.

Table 1. 5 Summary of Observational Study results conducted in Child Care Settings

Author	Purpose	Data Collection Methods	Results	Limitations
Taylor et al. (2008)	Explore understanding, knowledge and actions of child-care centers' staff regarding enteric illness and outbreaks and identify challenges staff encounter while managing them.	40 participants forming 5 focus groups for interview.	Childcare facility staff members were well informed about each disease, and appeared to be prepared to take the appropriate actions when needed. However, it was pointed out that time and budget constraints may result in less than optimal responses in real world situations.	Study restricted to one geographic area in one province in Canada.
Enke et al. (2007)	Identify demographic characteristics, food safety and other practices that influence training and decisions made by child-care directors and staff	Questionnaires mailed to and received from 118 child-care center directors from Iowa and Texas that met the study criteria with a response rate of 63%.	Educational initiatives in food safety were provided by less than one third of the surveyed facilities (31%); 83% of facilities used bleach to clean surfaces and equipment. Hand sanitizer was available in 45% of employee restrooms.	Self-reported practices

Table 1.5 Continued

Author	Purpose	Data Collection Methods	Results	Limitations
Kotch et al. (2007)	Determine if installation of equipment for diaper-changing, hand washing, and food preparation specifically designed to reduce transmission of infectious agents would result in a decrease in the rate of diarrheal illness.	23 pairs of centers were matched, with the intervention being installation of new diaper changing, hand washing, and food preparation equipment. Data on diarrheal illness incidence was collected by phone call to families.	Centers receiving the intervention demonstrated reduced diarrheal illness frequencies compared to control facilities (0.90 vs. 1.58 illnesses per 100 child-days) and lower illness absence rates for staff (0.77% 1.73%)	Classrooms randomly matched without stratifying for classroom type. Cost of equipment purchase and installation, averaging $10,385 per classroom, may be prohibitive for many child-care facilities.
Sandora et al. (2005)	Determine whether a multifactorial campaign centered on increasing alcohol-based hand sanitizer use and hand-hygiene education reduces illness transmission in the home of families with children who were enrolled in out-of-home child care.	Cluster randomized controlled trial of 292 families with children enrolled in out-of-home child care in 26 child-care centers. Data collected by telephone interview.	Training on use of alcohol-based hand sanitizers reduced the transmission of GI illness among children. The incidence rate ratio between treatment and control groups was 0.41 (95% confidence interval: 0.19-0.90)	Participants mainly families with high educational levels and incomes so results not generalizable to families of different cultural backgrounds or lower socioeconomic status. Documentation of illness based on symptom reported by caregivers rather than clinical confirmation of infection.

Table 1.5 Continued

Author	Purpose	Data Collection Methods	Results	Limitations
Chapman et al. (1995)	Evaluate if different hygiene practices were present in group and family child-care homes, and practices associated with frequency and type of illness prevalence in enrolled children.	Cross-sectional survey with self-administered questionnaires mailed to child-care homes. 204 licensed family and 137 group day-care homes in Washtenaw County MI surveyed.	Group day care homes had higher illness prevalence (2.7illnesses/home) than family day care homes (0.73 illnesses/home). Children who did not wash hands after diaper changing or before meals had significantly greater risk of illness [odds ratio=2.99, (95% confidence interval 1.25-7.14); and odds ratio=2.46 (95% confidence interval 0.95 to 6.39), respectively].	Non-response group may have induced selection bias The presence of illness was determined by the signs and symptoms of ill children displayed to the child care provider instead of consulting doctors. Asymptomatic cases will not be detected. Misclassification and recall bias could have occurred if day care providers did not report symptoms accurately.
Kotch et al. (1994)	Develop a feasible, multicomponent hygienic intervention and measure its impact.	Telephone interview used for the diarrheal and respiratory symptoms. Classroom observation and hygiene event sampling from assessing physical environment of classroom or collecting information about targeted behavior. 24 day-care centers were involved.	Intervention improved the mean incidence of all diarrhea moderately (3.99 vs 4.73 episodes/child-year in treatment vs. control centers). Greatest reductions were seen in younger children of less than 24 months (0.67 vs 1.33 episodes of severe diarrhea/child-year in treatment vs. control centers) and in newer centers of less than 6.5 years (0.38 vs 1.18 episodes of severe diarrhea/child-year in treatment vs. control) centers)	Study conducted in larger centers caring for younger children so results not generalizable to smaller centers or family day-care homes.

Table 1.6 State Requirements for Frequency of Environmental Health Inspections by Facility Type

Frequency of Inspection	Type of Child-Care Facility					
	Child-Care Center	**Number of States**	**Small Family Child-Care Homes**	**Number of States**	**Large/Group Family Child-Care Homes**	**Number of States**
3x year+	NV, TN	2	TN	1	TN	1
3x year	AR, FL, OK	3	AR, OK	2	OK	1
2x year	MS, MO, OH, RI, SC, UT, VA, WI, WY	9	FL, GA, MS, MO, NV, UT, WY	7	AZ, FL, MS, MO, NV, OH, SC, UT, VA, WY	10
1x year	AZ, DE, DC, HI, IL, IN, IA, KS, KY, LA, ME, MD, MA, MI, MT, NE, NH, NM, NC, ND, OR, PA, SD, TX, VT, WA, WV	26	DE, DC, HI, IL, IN, KY, ME, MD, MA, NE, NH, NM, NC, VT, WV, WI	16	DE, HI, IL, IN, KS, MA, MI, NE, NH, NM, ND, OR, PA, RI, SD, TX, WV	17
1x 2 years	AL, AK, CT, MN, NY	5	AL, AK, MN, NY, OR, RI, TX, WA	8	AL, AK, CT, MN, NY	5
1x 3 years	NJ	1	CA, CT	2	CA	1
>1x 3 years	No states	0	MT	1	MT	1
Other	CA, CO, GA	3	CO, IA	2	CO, GA, IA	3

Table 1.7 Summary of Regulations related to the Management of GI Illness in Child-Care Centers and Small and Large Day-Care Homes

Child-Care Centers (AL-MO)	AL	AK	AZ	AR	CA	CO	CT	DE	DC	FL	GA	HI	ID	IL	IN	IA	KS	KY	LA	ME	MD	MA	MI	MN	MS	MO
Licensed by State	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X
INSPECTION																										
Before Licensing	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X
At License Renewal	X	X	X					X	X	X		X		X	X	X	X	X	X	X		X	X		X	X
Routine Compliance	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X		X	X	X	X	X	X	X	X	X
3+/year						X					X															
3x /year				X		X				X	X															
2x /year						X					X														X	X
1x /year			X			X		X	X		X	X		X	X	X	X	X	X	X	X	X	X			
1x every 2 years	X	X				X	X																	X		
1x every 3 years						X																				
>1x every 3 years					X																					
STAFF TRAINING																										
First Aid	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	X	X		X	X	X	X	X	X	
CPR	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X		X	X	X	X	X	X	X	X	
Prevent spread of communicable diseases	X	X	X		X	X	X	X	X	X	X			X	X	X					X	X				
Care of ill child					X		X				X			X							X					
CHILD EXAM/ IMMUNIZATION																										
Physical exam required					X	X	X	X	X	X		X		X	X	X	X				X	X	X	X		X
Immunization pre-enrollment	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X

Table 1.7 Continued

Child-Care Centers (AL-MO)	**AL**	**AK**	**AZ**	**AR**	**CA**	**CO**	**CT**	**DE**	**DC**	**FL**	**GA**	**HI**	**ID**	**IL**	**IN**	**IA**	**KS**	**KY**	**LA**	**ME**	**MD**	**MA**	**MI**	**MN**	**MS**	**MO**
FOOD HANDLING																										
FDA Food Code	X	X		X			X								X		X	X	X			X		X	X	
Nutrient values of meals	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X
Infant feeding requirements	X	X	X	X	X	X	X	X	X		X	X		X	X	X	X	X	X	X	X	X	X	X	X	X
Parent/Guardian can provide own food	X	X	X			X	X	X				X				X		X	X	X	X	X		X	X	
Centers must provide meals	X													X	X								X			X
Parent/Guardian allowed to bring food for special events														X		X									X	
HAND-HYGIENE																										
Required toileting/hand washing activities	X	X			X		X				X			X			X					X				X
Specify when staff must wash hands	X	X	X	X			X	X	X	X	X			X	X	X	X	X		X	X	X	X	X	X	X
Required locations/ number sinks for staff		X	X	X	X		X	X						X	X			X			X					X
Specify when child must wash hands	X		X	**X**	X		X	X	X	X	X			X	X	X	X	X		X	X	X	X	X	X	X
Required locations/number sinks for children	X		X		X		X	X		X	X	X		X	X	X	X	X		X	X	X	X	X	X	X
Environmental health inspection required	X	X	X	X		X	X		X	X				X			X	X	X	X	X	X	X	X	X	X

Table 1.7 Continued

Child-Care Centers (AL-MO)	**AL**	**AK**	**AZ**	**AR**	**CA**	**CO**	**CT**	**DE**	**DC**	**FL**	**GA**	**HI**	**ID**	**IL**	**IN**	**IA**	**KS**	**KY**	**LA**	**ME**	**MD**	**MA**	**MI**	**MN**	**MS**	**MO**
ILL CHILDREN																										
Exclude mildly ill children	X		X	X	X	X	X	X	X	X	X	X		X	X			X	X			X		X	X	X
Admit mildly ill children		X										X		X		X						X				X
DIAPERING																										
Required for discarding soiled diapers																										
Sanitize diaper station after each use	X		X	X	X		X	X	X	X	X	X		X	X	X	X	X		X	X	X		X	X	X
Exclusive sinks for diaper areas	X		X	X	X		X	X	X	X	X			X	X		X	X		X		X			X	X
No diapering sinks used for food preparation.			X				X	X			X												X		X	
Wear gloves to change diapers					X																				X	X

Table 1.7 Continued

Child-Care Centers (MT-WY)	MT	NE	NV	NH	NJ	NM	NY	NC	ND	OH	OK	OR	PA	RI	SC	SD	TN	TX	UT	VT	VA	WA	WV	WI	WY
Licensed by State	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
INSPECTION																									
Before Licensing	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
At License Renewal	X	X	X	X	X	X	X		X	X		X	X		X	X	X		X	X	X	X	X		X
Routine Compliance	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
3+ /year			X														X								
3x/year											X														
2x/year										X				X	X				X		X			X	X
1x/year	X	X		X		X		X	X			X	X			X		X		X		X	X		
1x every 2 years							X																		
1x every 3 years					X																				
>1x every 3 years																									
STAFF TRAINING																									
First Aid	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
CPR	X	X	X	X	X	X		X	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X	X
Prevent spread of communicable diseases						X	X			X	X				X	X	X	X				X		X	
Care of ill child										X							X	X						X	
CHILD EXAM/ IMMUNIZATION																									
Physical exam required			X	X	X		X		X	X			X	X	X			X	X		X		X	X	
Immunization pre-enrollment	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Table 1.7 Continued

Child-Care Centers (MT-WY)	MT	NE	NV	NH	NJ	NM	NY	NC	ND	OH	OK	OR	PA	RI	SC	SD	TN	TX	UT	VT	VA	WA	WV	WI	WY
FOOD HANDLING																									
FDA Food Code		X			X				X	X	X			X		X	X						X		
Nutrient value of meals	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Infant feeding requirements	X	X	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Parent/Guardian can provide own food	X		X	X	X			X	X	X	X	X	X	X	X	X		X	X	X	X	X		X	X
Centers must provide meals																							X		
Parent/Guardian allowed to bring food for special events																						X			
HAND-HYGIENE																									
Required toileting/hand washing activities		X	X	X								X							X				X	X	
Specify when staff must wash hands	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X
Required locations/number sinks for staff	X		X			X								X	X						X				
Specify when child must wash hands	X	X	X	X	X	X	X	X		X	X	X	X	X	X		X	X	X	X	X	X	X	X	
Required locations/number sinks for children	X	X	X	X	X	X	X		X		X	X	X	X	X		X	X	X	X	X	X	X	X	X
Environmental health inspection required	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	X	X	X		X		X		X

Table 1.7 Continued

Child-Care Centers (MT-WY)	**MT**	**NE**	**NV**	**NH**	**NJ**	**NM**	**NY**	**NC**	**ND**	**OH**	**OK**	**OR**	**PA**	**RI**	**SC**	**SD**	**TN**	**TX**	**UT**	**VT**	**VA**	**WA**	**WV**	**WI**	**WY**
ILL CHILDREN																									
Exclude mildly ill children	X			X	X	X	X	X		X		X	X	X	X			X	X	X	X	X	X	X	X
Admit mildly ill children	X						X	X	X			X	X	X	X				X			X		X	X
DIAPERING																									
Required for discarding soiled diapers																									
Sanitize diaper station after each use	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	
Exclusive sinks for diaper areas	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
No diapering sinks used for food preparation							X					X		X		X			X					X	
Wear gloves to change diapers	X		X		X							X		X		X			X	X				X	

Table 1.7 Continued

Small Family Day-Care Homes (AL-MO)	**AL**	**AK**	**AZ**	**AR**	**CA**	**CO**	**CT**	**DE**	**DC**	**FL**	**GA**	**HI**	**ID**	**IL**	**IN**	**IA**	**KS**	**KY**	**LA**	**ME**	**MD**	**MA**	**MI**	**MN**	**MS**	**MO**
Licensed by State	X	X		X	X	X	X	X	X	X	X	X		X	X	X	X	X		X	X	X	X	X	X	X
INSPECTION																										
Before Licensing	X	X		X	X	X	X	X	X	X	X	X		X	X			X		X	X	X		X	X	X
At License Renewal	X	X						X	X	X		X		X	X			X		X		X		X	X	X
Routine Compliance	X	X		X	X	X	X	X	X	X	X	X		X	X	X		X		X	X	X			X	X
3+/year						X																				
3x/year				X		X																				
2x/year						X				X	X														X	X
1x/year						X		X	X			X		X	X			X		X	X	X				
1x every 2 years	X	X				X																		X		
1x every 3 years					X	X	X																X			
>1x every 3 years																										
STAFF TRAINING																										
First Aid	X	X			X	X	X	X	X	X	X	X		X	X	X	X	X		X	X	X	X	X	X	
CPR	X	X		X	X	X	X		X	X	X	X		X	X			X		X	X	X	X	X	X	
Prevent spread of communicable diseases	X				X	X		X		X	X				X											
Care of ill child																										
CHILD EXAM/ IMMUNIZATION																										
Physical exam required	X					X	X	X	X	X		X		X	X	X		X			X	X	X			X
Immunization pre-enrollment	X	X		X	X	X	X	X	X	X	X	X		X	X	X	X	X		X	X	X	X	X	X	X

Table 1.7 Continued

Small Family Day-Care Homes (AL-MO)	**AL**	**AK**	**AZ**	**AR**	**CA**	**CO**	**CT**	**DE**	**DC**	**FL**	**GA**	**HI**	**ID**	**IL**	**IN**	**IA**	**KS**	**KY**	**LA**	**ME**	**MD**	**MA**	**MI**	**MN**	**MS**	**MO**
FOOD HANDLING																										
FDA Food Code																										
Nutrient value of meals	X	X		X		X		X	X	X	X	X		X		X		X			X	X	X	X	X	X
Infant feeding requirements	X	X		X		X	X	X	X		X	X		X				X		X	X	X	X	X	X	X
Parent/Guardian can provide own food		X		X				X	X			X		X		X				X		X	X	X		
Centers must provide meals	X										X														X	X
Parent/Guardian allowed to bring food for special events																									X	
HAND-HYGIENE																										
Required toileting/hand washing activities		X												X												X
Specify when staff must wash hands	X	X		X		X	X	X	X	X	X			X			X	X		X	X	X	X	X	X	X
Required locations/number sinks for staff		X		X				X				X			X		X	X		X			X		X	X
Specify when child must wash hands	X	X		X		X		X	X	X				X	X			X		X	X	X	X	X	X	X
Required locations/number sinks for children																										
Environmental health inspection required		X							X											X	X				X	X

Table 1.7 Continued

Small Family Day-Care Homes (AL-MO)	**AL**	**AK**	**AZ**	**AR**	**CA**	**CO**	**CT**	**DE**	**DC**	**FL**	**GA**	**HI**	**ID**	**IL**	**IN**	**IA**	**KS**	**KY**	**LA**	**ME**	**MD**	**MA**	**MI**	**MN**	**MS**	**MO**
ILL CHILDREN																										
Exclude mildly ill children	X			X					X							X					X					
Admit mildly ill children							X	X		X				X				X				X				
DIAPERING																										
Required for discarding soiled diapers	X							X	X		X			X			X			X	X		X	X	X	X
Must sanitize diaper station after each use	X			X		X	X	X	X	X	X			X			X	X		X			X	X		X
Exclusive sinks for diaper areas																										
No diapering sinks used for food prep.	X					X		X	X	X										X		X	X	X		X
Wear gloves to change diapers	X								X																	

Table 1.7 Continued

Small Family Day-Care Homes (MT-WY)	**MT**	**NE**	**NV**	**NH**	**NJ**	**NM**	**NY**	**NC**	**ND**	**OH**	**OK**	**OR**	**PA**	**RI**	**SC**	**SD**	**TN**	**TX**	**UT**	**VT**	**VA**	**WA**	**WV**	**WI**	**WY**
Licensed by State	X	X	X	X		X	X	X	X		X	X	X	X	X		X	X	X	X		X	X	X	X
INSPECTION																									
Before Licensing		X	X	X		X	X	X			X	X		X			X	X	X	X		X	X	X	X
At License Renewal	X		X	X		X						X		X			X		X	X		X	X		X
Routine Compliance	X	X	X	X		X	X	X			X			X			X	X	X	X		X	X	X	X
3+ year																	X								
3x/year											X														
2x/year			X																X						X
1x/year		X		X		X		X												X			X	X	
1x every 2 years							X					X	X	X				X				X			
1x every 3 years																									
>1x every 3 years	X																								
STAFF TRAINING																									
First Aid	X	X	X	X		X		X	X		X	X	X	X			X	X	X			X	X		X
CPR	X	X	X	X		X		X	X		X	X		X			X	X	X	X		X		X	X
Prevent spread of communicable diseases						X					X														
Care of ill child																									
CHILD EXAM/ IMMUNIZATION																									
Physical exam required			X	X			X	X	X				X	X				X	X				X	X	
Immunization pre-enrollment	X	X	X	X		X	X	X	X		X	X	X	X			X	X	X	X		X	X	X	X

Table 1.7 Continued

Small Family Day-Care Homes (MT-WY)	**MT**	**NE**	**NV**	**NH**	**NJ**	**NM**	**NY**	**NC**	**ND**	**OH**	**OK**	**OR**	**PA**	**RI**	**SC**	**SD**	**TN**	**TX**	**UT**	**VT**	**VA**	**WA**	**WV**	**WI**	**WY**
FOOD HANDLING																									
FDA Food Code																									
Nutrient value of meals	X	X	X	X		X		X			X	X	X	X			X	X	X	X		X	X	X	X
Infant feeding requirements	X	X	X	X		X	X	X	X		X	X	X	X			X	X	X			X	X	X	X
Parent/Guardian can provide own food			X	X		X	X						X	X				X	X			X		X	X
Centers must provide meals								X			X						X								
Parent/Guardian allowed to bring food for special events																									
HAND-HYGIENE																									
Required toileting/hand washing activities														X									X		
Specify when staff must wash hands	X	X	X	X		X	X	X	X		X	X	X	X			X	X	X	X		X	X	X	X
Required locations/number sinks for staff	X	X	X	X				X			X	X	X	X			X	X	X	X		X	X	X	X
Specify when child must wash hands	X		X	X		X	X	X			X	X	X	X			X	X	X	X		X	X	X	
Required locations/number sinks for children																									
Environmental health inspection required			X	X		X											X		X						X

Table 1.7 Continued

Small Family Day-Care Homes (MT-WY)	**MT**	**NE**	**NV**	**NH**	**NJ**	**NM**	**NY**	**NC**	**ND**	**OH**	**OK**	**OR**	**PA**	**RI**	**SC**	**SD**	**TN**	**TX**	**UT**	**VT**	**VA**	**WA**	**WV**	**WI**	**WY**
ILL CHILDREN																									
Exclude mildly ill children	X													X				X						X	X
Admit mildly ill children								X					X				X		X	X				X	
DIAPERING																									
Required for discarding soiled diapers	X	X	X	X			X	X	X		X		X	X			X	X	X			X	X	X	
Sanitize diaper station after each use	X		X	X		X	X		X		X		X	X				X	X			X	X	X	X
Exclusive sinks for diaper areas																									
No diapering sinks used for food preparation	X		X	X			X	X	X				X	X			X	X	X	X		X			X
Wear gloves to change diapers				X								X												X	

Table 1.7 Continued

Large Group Day-Care Homes (AL-MO)	AL	AK	AZ	AR	CA	CO	CT	DE	DC	FL	GA	HI	ID	IL	IN	IA	KS	KY	LA	ME	MD	MA	MI	MN	MS	MO
Licensed by State	X	X	X		X	X	X	X		X	X	X		X	X	X	X					X	X	X	X	X
INSPECTION																										
Before Licensing	X	X	X		X	X	X	X		X	X	X		X	X		X					X	X	X	X	X
At License Renewal	X	X	X					X		X		X		X	X		X					X	X	X	X	X
Routine Compliance	X	X	X		X	X	X	X		X	X	X		X	X	X						X	X		X	X
3+/year						X					X															
3x/year						X					X															
2x/year			X			X				X	X														X	X
1x/year						X		X			X	X		X	X		X					X	X			
1x every 2 years	X	X				X	X																	X		
1x every 3 years					X	X																				
>1x every 3 years																										
STAFF TRAINING																										
First Aid	X	X	X		X	X	X	X		X	X	X		X	X	X	X					X	X	X	X	
CPR	X	X	X		X	X				X	X	X		X	X							X	X	X	X	
Prevent spread of communicable diseases	X		X		X	X	X	X		X	X				X											
Care of ill child			X				X				X															
CHILD EXAM/ IMMUNIZATION																										
Physical exam required						X	X	X		X		X		X	X	X	X					X	X			X
Immunization pre-enrollment	X	X	X		X	X	X	X		X	X	X		X	X	X	X					X	X	X	X	X

Table 1.7 Continued

Large Group Day-Care Homes (AL-MO)	AL	AK	AZ	AR	CA	CO	CT	DE	DC	FL	GA	HI	ID	IL	IN	IA	KS	KY	LA	ME	MD	MA	MI	MN	MS	MO
FOOD HANDLING																										
Follows FDA Food Code																										
Nutrient value of meals	X	X	X			X	X	X		X	X	X		X		X	X					X	X	X	X	X
Infant feeding requirements	X	X	X			X		X			X			X			X					X	X	X	X	X
Parent/Guardian can provide own food		X	X				X	X			X	X		X		X						X	X	X		
Centers provide meals	X																								X	X
Parent/Guardian allowed to bring food for special events																									X	
HAND HYGIENE																										
Required toileting/ hand-washing activities		X					X				X			X												X
Specify when staff must wash hands	X	X	X			X	X	X		X	X											X	X	X	X	X
Required locations/ number sinks for staff			X				X	X			X				X								X			X
Specify when child must wash hands	X	X	X			X	X	X		X	X				X							X	X	X	X	X
Required locations/ number sinks for children																										
Environmental health inspection required		X					X										X								X	X

Table 1.7 Continued

Large Group Day-Care Homes (AL-MO)	AL	AK	AZ	AR	CA	CO	CT	DE	DC	FL	GA	HI	ID	IL	IN	IA	KS	KY	LA	ME	MD	MA	MI	MN	MS	MO
ILL CHILDREN																										
Exclude mildly ill children	X		X				X					X				X										X
Admit mildly ill children								X		X		X		X								X				X
DIAPERING																										
Required for discarding soiled diapers	X		X				X	X						X			X						X	X	X	X
Sanitize diaper station after each use	X		X			X	X	X		X	X			X			X						X	X		X
Exclusive sinks for diaper areas																										
No diapering sinks used for food prep.	X		X			X	X	X		X	X											X	X	X		X
Wear gloves to change diapers	X																									

Table 1.7 Continued

Large Group Day-Care Homes (MT-WY)	**MT**	**NE**	**NV**	**NH**	**NJ**	**NM**	**NY**	**NC**	**ND**	**OH**	**OK**	**OR**	**PA**	**RI**	**SC**	**SD**	**TN**	**TX**	**UT**	**VT**	**VA**	**WA**	**WV**	**WI**	**WY**
Licensed by State	X	X	X	X		X	X		X	X	X	X	X	X	X	X	X	X	X		X		X		X
INSPECTION																									
Before Licensing		X	X	X		X	X		X	X	X	X	X	X	X	X	X	X	X		X		X		X
At License Renewal	X	X	X	X		X	X		X	X		X	X	X	X	X	X		X		X		X		X
Routine Compliance	X	X	X	X		X	X		X	X	X	X	X	X	X	X	X	X	X		X		X		X
3+/year																	X								
3x/year											X														
2x/year			X							X					X				X		X				X
1x/year		X		X		X			X			X	X	X		X		X					X		
1x every 2 years							X																		
1x every 3 years																									
>1x every 3 years	X																								
STAFF TRAINING																									
First Aid	X	X	X	X		X			X	X	X	X	X	X	X	X	X	X	X		X				X
CPR	X	X	X	X		X			X	X	X	X		X	X	X	X	X	X				X		X
Prevent spread of communicable diseases						X				X	X														
Care of ill child																									
CHILD EXAM/ IMMUNIZATION																									
Physical exam required			X	X			X		X	X			X	X	X			X	X		X		X		
Immunization pre-enrollment	X	X	X	X		X	X		X	X	X	X	X	X	X	X	X	X	X		X		X		X

Table 1.7 Continued

Large Group Day-Care Homes (MT-WY)	MT	NE	NV	NH	NJ	NM	NY	NC	ND	OH	OK	OR	PA	RI	SC	SD	TN	TX	UT	VT	VA	WA	WV	WI	WY
FOOD HANDLING																									
FDA Food Code																									
Nutrient value of meals	X	X	X	X		X			X	X	X	X	X	X	X	X	X	X	X		X		X		X
Infant feeding requirements	X	X	X	X		X	X		X	X	X	X	X	X	X	X	X	X	X		X		X		X
Parent/Guardian can provide own food			X	X		X	X		X	X		X	X	X	X	X		X	X		X		X		X
Centers must provide meals											X						X								
Parent/Guardian allowed to bring food for special events																									
HAND-HYGIENE																									
Required toileting/hand washing activities												X		X									X		
Specify when staff must wash hands	X	X	X	X		X	X		X	X	X	X	X	X	X	X		X	X		X				X
Required locations/number sinks for staff	X	X							X		X	X	X	X	X			X							X
Specify when child must wash hands	X		X	X		X	X			X	X	X	X	X		X		X	X		X				
Required locations/number sinks for children																									
Environmental health inspection required			X	X		X			X	X		X			X	X	X		X				X		X

Table 1.7 Continued

Large Group Day-Care Homes (MT-WY)	**MT**	**NE**	**NV**	**NH**	**NJ**	**NM**	**NY**	**NC**	**ND**	**OH**	**OK**	**OR**	**PA**	**RI**	**SC**	**SD**	**TN**	**TX**	**UT**	**VT**	**VA**	**WA**	**WV**	**WI**	**WY**
ILL CHILDREN																									
Exclude mildly ill children	X								X	X		X	X	X	X			X			X		X		X
Admit mildly ill children													X			X			X						
DIAPERING																									
Required for discarding soiled diapers	X	X	X	X			X		X	X	X		X	X			X	X	X		X		X		
Sanitize diaper station after each use	X		X	X		X	X		X	X	X	X	X	X				X	X		X		X		X
Exclusive sinks for diaper areas																									
No diapering sinks used for food prep.	X		X	X			X		X			X	X	X			X	X	X		X				X
Wear gloves to change diapers						X								X											

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CHAPTER 2 Microbiological Analysis of Surfaces and Workers' Hands in Child Care Facilities in North and South Carolina

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Abstract

About 60% of U.S. children, age 5 and younger, spend time in childcare. For many reasons, these children are at increased risk of enteric disease, including those that are foodborne. The purpose of this study was evaluate the microbiological quality of environmental samples collected from representative childcare centers located in North and South Carolina, USA. Forty childcare facilities were visited; 31 (77.5%) were centers and 9 (22.5%) were home-based. Environmental samples corresponding to common surfaces (faucets, toys, refrigerators, diaper changing areas) and the hands of care providers and food workers were collected. These were analyzed for total aerobic bacterial count (APC), total coliforms, and generic *Escherichia coli,* as well as for *Shigella* spp, *Salmonella*, *E. coli* O157:H7, *Campylobacter jejuni*, and genogroups I and II human norovirus (NoV). A total of 326 samples were evaluated: 74 hand rinsates, 83 environmental samples taken from regular (flat) surfaces (FlS; 100 cm^2), and 169 environmental samples taken from irregular surfaces (IrS; e.g., faucets, toys). For hands, median APC and coliform counts were 4.5 and 1.8 log_{10}

CFU/hand, respectively. For common surfaces, median APC was 2.2 (FlS) and 2.7 (IrS) $\log_{10}$ CFU/surface sampled, with median coliform counts 1.3 $\log_{10}$ CFU/surface. Coliforms were detected in 53 of 326 (16.2%) samples with counts ranging from 1 to >4.3 $\log_{10}$ CFU per sample. When elevated coliform and APC counts did occur (defined as >5.3 $\log_{10}$ CFU per sample), they were mostly associated with hands (17.6%). Generic *E. coli* counts were below the assay detection limits (<1 $\log_{10}$ CFU per sample) for all but one hand sample. No samples were positive for any of the four bacterial pathogens. Seven of the samples (2.1%) tested presumptively positive for genogroup I NoV and 41 (12.6%) for genogroup II NoV, although only four of these samples could be confirmed for NoV contamination using dot blot hybridization. The relative absence of pathogens and generic *E. coli* in these samples suggests that the childcare facilities sampled in this study managed fecal contamination well.

Introduction

Organized childcare is an integral part of our society. In the U.S alone, 61% of children (from birth to six years of age), who were not enrolled in kindergarten, had spent time in non-parental childcare in 2005 (Child Trends Databank, 2005). Not only is the number of children participating in childcare important, but so is the amount of time spent in caring for children outside the home. In the U.S, children with employed mothers are estimated to spend an average of 25 to 33 hours in childcare per week; children with unemployed mothers averaged 19 hours per week (Lynda 2010).

For a number of reasons, including the need for diapering, the close contact between children and their shared belongings, and the higher prevalence of mild respiratory and

gastrointestinal illness in this population relative to adults, there are many opportunities for the transmission of diseases in the childcare environment. In fact, it is estimated that U.S children cared for outside the home are between 2.3 and 3.5 times more likely to experience a diarrheal disease episode than are those cared for in their own homes (Lu et al, 2004). The total annual cost of diarrheal disease in children under age 10 may be as high as $2.3 billion (Buzby, 2001).

There are many agents that can be transmitted in the childcare environment, including pathogenic bacteria, viruses, and protozoa. According to FoodNet, *Salmonella*, *Campylobacter*, *Shigella*, and shiga-toxin producing *Escherichia coli* (STEC) constitute the top four bacterial pathogens causing the highest numbers of laboratory confirmed gastrointestinal disease cases in children under the age of five years (CDC, 2012). A comprehensive review of 75 enteric disease outbreaks associated with childcare settings (Lee and Greig, 2008) concluded that bacterial and viral agents together were responsible for 93.4% (70/75) of these outbreaks, while 6.6% (5/75) were attributed to parasitic protozoa. Bacteria and virus were responsible for an equal amount of outbreaks (35 each); viral agents were responsible for more cases (55.7%). Other investigators have reported similar findings (Ethelberg et al., 2006; Rosenfeldt et al., 2005). Viral outbreaks are caused by rotaviruses, human noroviruses, and astroviruses (Lyman et al., 2009; Akihara et al., 2005); bacterial outbreaks are usually caused by STEC strains and *Shigella spp*. (Lee and Grieg, 2008). Recent data also suggest that childcare attendance is a risk factor for salmonellosis (Jones et al., 2006).

A number of environmental studies have sought to characterize microbiological contamination in the childcare environment. Early studies focused on using general microbial tests such as the aerobic plate count or coliform count as indicators of overall microbiological load on hands and surfaces, as well as fecal coliforms to indicate recent contamination with fecal matter (Ekanem et al., 1983; Weniger et al., 1983; Van et al., 1990; LaBorde et al., 1993). Relatively fewer studies have looked for evidence of specific pathogen contamination, although the advent molecular-based detection methods are making these studies more feasible. For example, Boone and Gerba (2005) found evidence of influenza A virus contamination in environmental samples obtained from childcare centers, while Wilde et al (1992) identified rotavirus contamination associated with about 30% of the environmental samples taken during a diarrheal outbreak in two childcare centers. Similar studies seeking to identify bacterial pathogens in childcare environments have yet to be done.

As part of a broader project, the purpose of which was to identify risk factors associated with enteric infection in the childcare environment, the purpose of this study was to collect microbiological data from environmental samples taken from representative childcare centers located in the U.S. States of North Carolina and South Carolina.

Materials and Methods

Sampling sites and surface selections

Eighteen North Carolina and 22 South Carolina child-care facilities were visited from September, 2010 through February, 2011, of which 31 (77.5%) were classified as centers and 9 (22.5%) as homes. Environmental samples were collected (in duplicate) from common

surfaces (faucets, toys, refrigerators, diaper changing areas etc.) and the hands of care providers and food workers. The average number of samples taken from each facility was 16 (range of 10-20 samples per facility). To maintain confidentiality of the results, all sampling data was coded and the analyst conducting the microbiological testing was blinded to sample identity.

Environmental Sampling Protocol

For surface sampling, the 3M Swab sampler-Letheen Broth (3M, St Paul, MN) was used. For flat surfaces (e.g. diaper changing surfaces, food serving areas), a 10 cm x 10 cm square area was demarcated using a disposable cardboard template (Weber Scientific, Hamilton, NJ). If the surface was of irregular shape (e.g. a utensil, toy, faucet, etc.), swabbing was performed on the entire sample surface without the use of the template. Specifically, the swab head was pressed against the surface of its container to release excess moisture, rubbed slowly and thoroughly over the target area one time, reversing direction with each stroke. This procedure was repeated twice using different swabbing directions for each replicate. The swab was then deposited back in the Letheen broth, sealed, placed on ice, and prepared for shipment to the analytical laboratory. Each surface was sampled in duplicate using swabs immersed in 10 ml (for use in bacterial analysis) and 1 ml (for use in viral analysis) of Letheen broth. For hand sampling, the method described by Kampf *et al*. (2006) was used. Briefly, the distal phalanges (i.e., fingertips) of the left and right hand (one for bacterial analysis, one for viral analysis) were rubbed separately, including thumbs, for 1 min in a Petri dish (9 cm diameter) containing 10 ml of tryptic soy broth (Thermo Fisher Scientific, Lenexa, KS). The sampling fluid for each hand was then aseptically transferred in

its entirety to sterile capped plastic vials and shipped on ice to North Carolina State University. Microbiological analyses were initiated within 24 h (usually 12 to 18 h) of sample collection. A flow diagram of sample processing for microbiological testing is shown in Figure 2.1.

Enumerative Assays for Microbiological Indicators

One ml of each 10 ml swab solution was plated to 3M^{TM} Petrifilm^{TM} Aerobic Count Plates (3M, St Paul, MN) in accordance with manufacturer instructions, followed by incubation at 35°C for 48 h prior to enumeration. An additional 1 ml aliquot was plated to 3M^{TM} Petrifilm^{TM} *E.coli/*coliform Count Plates and incubated at 35°C for 24-48 h, according to manufacturer instructions. Coliform colonies were red or blue in color, while *E. coli* colonies appeared as blue in color, both of which were surrounded by gas bubbles in accordance with the manufacturer's interpretation guide. Results for all counts were reported as CFU per hand or per surface.

Presence/Absence Assays for Bacterial Pathogens

Samples were screened for four different bacterial pathogens: *Salmonella* spp, *Shigella* spp., *E. coli* O157:H7, and *Campylobacter jejuni*. Cultural enrichment of the first three pathogens was done by placing 2.5 ml of the 10 ml swab sample into 22.5 ml of Universal Pre-enrichment Broth (UPB, Difco Laboratories, Sparks, MD) followed by incubation at 37 ^{0}C for 24 h. Screening for *E. coli* O157:H7 was done using the Tecra^{TM} *E. coli* O157 Visual Immunoassay kit (3M) with the input of 1 ml UPB as per manufacturer instructions. For detection of *Salmonella*, 1 ml UPB was further transferred into Rapport-

Vassiliadis (RV) medium (Thermo-Fisher) which was incubated at 37 ^{0}C for 24 h, after which 1 ml was used in the Tecra™ *Salmonella* Visual Immunoassay kit (3M).

For detection of *Shigella* spp, UPB was streaked onto XLD agar (Difco Laboratories, Sparks, MD) and incubated at 35 ± 2^{0}C for 18-24 h; presumptively positive colonies were identified as red in color (Taylor, 1965). A 250 µl aliquot of UPB was also extracted for DNA isolation using a NucliSens™ EasyMag instrument (bioMerieux, Durham, NC) according to manufacturer instructions, with the extracted DNA reconstituted in the elution buffer (bioMerieux) to a total volume of 50 µl. This DNA was subjected to quantitative real-time PCR (qPCR) targeting the *ipaH* gene which encodes the invasion plasmid antigen H for *Shigella* spp. (Wang et al., 2007). Primer and probe sequences are provided in Table 2.1. The 25 µl reaction mixture consisted of 2 µl sample DNA, 0.4 µM of primers, 0.2 µM probe, 0.2 mM of each dNTP (dATP, dCTP, dGTP, and dTTP; Applied Biosystems, Foster City, CA), 0.5 µl of reference dye ROX (Invitrogen, Carlsbad, CA) and 15.5 µl DPEC-treated nuclease-free sterile water to a PCR reaction tube containing the concentrations of PCR buffer, $MgCl_2$ and enzyme mixture recommended by the manufacture of Plantium *Taq*™ polymerase (Invitrogen). Amplification and detection were carried out in optical-grade 48-well plates using a StepOne™ Real-Time PCR system (Applied Biosystems, Foster City, CA). The parameters for real-time PCR included an initial cycle of 95^{0}C for 2 min followed by 40 cycles of 95^{0}C for 10 sec, 55^{0}C for 20 sec and 72^{0}C for 20 sec. The optics value was collected at the extension stage of the PCR cycle.

For detection of *C. jejuni*, an additional 2.5 ml aliquot from the 10 ml swab sample was inoculated into Bolton Broth (OXOID LTD, Basingstoke, Hampshire, England) and

incubated at 42^0C for 48 h under microaerophilic conditions of 5% O_2, 10% CO_2, 85% N_2 (Hunt *et al*., 2001). After incubation, the Bolton broth was streaked onto Campy Cefex agar (Hardy Diagnostics, Santa Maria, CA) which was incubated micoaerophilically at 42^0C for 48 h. The plates were examined for typical growth which appeared as small, mucoid, grayish, flat colonies with irregular edges and no hemolytic patterns.

Processing of Environmental Samples for Detection of Human Noroviruses

For hand rinsates, 10 ml samples were supplemented to a total salt concentration of 5% NaCl, to which was added 1% (w/v) bovine serum albumin and 12% (w/v) polyethylene glycol (Sigma, St. Louis, MO). The solution was stored overnight at 4^0C with continuous shaking after which it was centrifuged at 18,500 *x g* for 20 min at 4^oC. The virus-containing pellet was recovered and reconstituted in 1 ml 1X phosphate buffered saline (MP Biomedicals, Solon, OH). This was then subjected to RNA extraction using the NucliSens™ EasyMag Instrument. The 1 ml environmental swab samples were extracted in their entirety for RNA isolation without prior PEG precipitation. Final RNA pellets were reconstituted in the elution buffer to a total sample volume of 25 µl.

Detection of Human Noroviruses in RNA Extracts Derived from Hand Rinses and Environmental Samples

Construction of an Internal Amplification Control (IAC).

An IAC is a non-target nucleotide sequence that is co-amplified simultaneously with the target sequence and can serve as a means by which to identify reaction failure (false

negative results) as a consequence of thermal cycler malfunction, faulty PCR mixture, insufficient DNA polymerase activity, and/or the presence of inhibitory substances in the sample matrix (Hoorfar et al., 2004).

A competitive IAC, constructed so as to be amplified by the same primers as the target, was produced using the composite primer technique (Siebert et al., 1992). Briefly, the IAC was synthesized by overlap extension PCR, after which the 230 bp PCR product was separated by gel electrophoresis, purified using the QIAquick PCR/Gel purification kit (Qiagen, Valencia, CA), and *in vitro* transcribed with the MEGAshortscript High Yield Transcription kit (Ambion, Austin,TX). A flow diagram in Figure 2.2 details this process. The final IAC concentration was determined using a Nanophotometer Pearl (Implen, Munchen, Germany) and its concentration for use in RT-qPCR was optimized by applying PCR (described below) to 10-fold serial dilutions of the concentrated IAC stock. The dilution displaying a consistent Ct value of about 30 was used to screen for RT-qPCR inhibition in all environmental sample extracts subject to NoV screening.

Amplification Reactions (Reverse-Transcription)-qPCR for Human Noroviruses.

Primers and probes for detection of genogroups I and II (GI and GII) NoV are provided in Table 2.1. For RT-qPCR amplifications, a 25 µl reaction mixture was prepared by adding the following to PCR reaction tubes containing the concentrations of reaction mixture (a buffer containing 0.4 µM of dNTP and 6mM $MgSO_4$) as recommended by the manufacture of SuperScript® III Platinum® One-Step Quantitative RT-PCR kit (Invitrogen): 2.5 µl of template RNA (derived from environmental samples); forward and reverse primers

and probes (1 µl of 0.4 µM GI forward primer; 1.5 µl of 0.6 µM GI reverse primer; 0.3 µl of 0.12 µM GI and IAC probe; 1.2µl of 0.48 µM GII forward/reverse primer; 1µl of 0.4 µM GII and IAC probe); 2 µl IAC (IAC probe sequence for GI and GII: TET-5'-ATCTCAGTTCGGTGTAGGTCGTTCGCTCC-3'-BHQ); 0.5 µl (GI) or 1.3 µl (GII) SuperScript® III Reverse Transcriptase/Platinum® Taq DNA polymerase mix; and 4.1 µl (GI) or 2.3 µl (GII) DPEC-treated nuclease-free sterile water Amplification and detection were carried out using the StepOne™ Real-Time PCR system. Reverse transcription was done at 50^0C for 15 min followed by enzyme inactivation at 95^0C for 2 min. This was immediately followed by 45 cycles of 95^0C for 10-15 sec, 54-55^0C for 20-30 sec and 72^0C for 15-30 sec. The optics value was collected at the annealing stage of PCR cycle. Because of the potential that amplification of the IAC could outcompete target amplification (especially if a sample had low levels of template), parallel amplifications were done without inclusion of the IAC. If the IAC amplification was inhibited or the IAC showed a Ct value greater than 35, the RNA template was diluted 10-fold and re-amplified.

Confirmation of Presumptively Positive Samples Using Dot blot Hybridization.

Presumptively positive signals for GI and GII NoV by RT-qPCR were confirmed by dot blot hybridization. Briefly, conventional RT-PCR amplifications were done using the same primer set as RT-qPCR (but without the fluorescently-labeled probe) on the RNA extracts corresponding to each presumptively positive sample. After amplification, 4 µl of denatured RT-PCR amplicons were spotted onto a nylon membrane (Roche Applied science, Mannheim, Germany), UV cross-linked, and the membrane pre-hybridized with Express

Hybridization Solution (Clontech Laboratories, Inc., Palo Alto, CA) for 30 min at 55°C. Hybridization was done for 2 h at 55°C in fresh solution supplemented with 100 pmol of tailed probes, which were the same as used in RT-qPCR but labeled with a 3' digoxigenin (DIG) label in place of the FAM and BHQ dyes, using the DIG-Oligonucleotide Tailing kit (Roche Applied Science, Mannheim, Germany) in accordance with manufacturer's instructions. After hybridization, the membrane was washed at room temperature for 10 min in 2X SSC, and for 30 min in 0.1X SSC. The membrane was processed for the enzyme-catalyzed colorimetric reaction using the DIG Nucleic Acid Detection kit (Roche). Specifically, visual detection of the DIG-labeled nucleic acids was done by treating the membranes with anti-digoxigenin alkaline phosphatase conjugate (anti-DIG-AP,) followed by 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium salt (NBT) which resulted in purple-blue precipitates. A flow diagram of dot blot hybridization is shown in Figure 2.3.

Statistical Analysis

Enumerative microbiological results were analyzed by calculating descriptive statistics (mean, median and mode) on the log-transformed data. Comparative analysis of indicator levels between childcare centers and homes was done using the nonparametric Mann-Whitney test using the StatCrunch 5.0 program (Integrated Analytics LLC, http://statcrunch.stat.ncsu.edu/). Statistical significance was determined at p=0.05.

Results and Discussion

A total of 652 environmental samples were collected in duplicate: 148 hand rinsates, 166 regular (flat) surfaces (100 cm^2), and 338 irregular surfaces (e.g., faucets, toys). Because sample collection was constrained by cost (estimated at $200/site), the sample size was not sufficient to allow inferences to all of child care facilities in NC and SC (12,728 licensed facilities in total with capacity of 655, 557 children as per the National Association for Regulatory Administration and National Child Care Information and Technical Assistance Center (2009). However, the sample size used in this study is considered adequate for describing the microbiological conditions (and observed food handling, hygiene and sanitation practices) in the participating facilities. Published observational studies on food handling practices or microbiological analysis in childcare facilities have relied on similar sample sizes (Clayton and Griffith, 2004 [n = 170]; Redmond et al., 2004 [n = 30]; Worsfold and Griffith, 1997 [n = 108]; Anderson et al., 2004 [n = 99]; Keswick et al., 1983 [n=25]; Staskel et al., 2007 [n=167]; Cosby et al., 2008 [n=1149]).

Aerobic plate counts (APC) and coliform counts were shown for all environmental sample types in Figures 2.4. In a study by Kennedy et al. (2005), the mean APC of worktops in domestic kitchens was 5.9 $\log_{10}$ CFU/100 cm^2, and the mean coliform count was 4.0 $\log_{10}$ CFU/100 cm^2. Henroid et al. (2004) suggested that mean $\log_{10}$ *Enterobacteriaceae* counts should not exceed 3.0 $\log_{10}$ CFU/100cm^2. In our study, the coliform counts for all regular surface samples' (n=83) fell within that limit (Figure 2.4). When hands samples are included in the analysis, coliforms were detected in 53 of 326 (16.2%) samples, which is consistent with the prevalence rate (24.7%) reported by Cosby *et al* (2007) and much lower than those

(79.6%) associated with tabletops and dishcloth in restaurants and bars (Yepiz-Gomez *et al.*, 2006). When elevated coliform (>10 cfu/surface or hand) and APC (>$2x10^5$ cfu/surface or hand) counts did occur, they were mostly associated with hands (elevated coliform count frequencies: 27% hand, 15.4% irregular surface and 8.4% regular surface; elevated APC frequencies: 17.6% hands, 5.9% irregular surface and 2.4% regular surface). The distributions of the APC and coliform count data as a function of environmental sample type are shown in histogram format in Figures 2.4. For APC data, there was more of a left skew to the data produced from hand samples relative to surface samples, suggesting higher levels of contamination on hands. This can be explained by the fact that hands are usually involved in more tactile events (at least, relative to a particular surface), and that as living tissue, they may be a better environment to support the survival of bacteria. However, the level and distribution of coliforms was more or less the same when comparing surfaces to hands, suggesting that both hand and surface sanitation were relatively efficient in the childcare facilities investigated.

The levels of generic *E. coli* were below assay detection limits (<10 CFU per sample) for all but one (hand) sample (2.87 $\log_{10}$ cfu/hand). This result is contradictory to other studies. For example, Ekanem et al (1983) recovered fecal coliforms in 32% of hand samples and 36% of environmental samples collected from 5 childcare centers. Van et al (1991) also isolated fecal coliforms from 46% of toy samples and 17% of hand samples from 6 childcare centers. However, in both of these studies, samples were collected during a diarrheal disease outbreak, whereas our samples were taken randomly and sampling was in no way associated with outbreaks. However, the single *E. coli* positive hand sample that we

detected may indicate that the potential for fecal contamination is greater for hands than it is for environmental samples. Indeed, failure to implement proper hand hygiene practices is probably the single most common reason for diarrheal diseases and contributes to many outbreaks of diarrhea among children and caregivers/teachers in childcare settings (Morrow et al., 1991, American Academy of Pediatrics, 2011A).

The APC and coliform levels for the diaper changing area (n=51) were not significantly different (*p*-value: 0.1745 for APC and 0.9287 for coliform) when compared to levels observed in the food serving area (n=16). There was an expectation that the diaper changing area should have higher bacterial counts than other areas; nonetheless, that was not the case in our study. Cosby et al. (2008) also observed low APC (1.58 $\log_{10}$ cfu/50 cm^2) in association with the diapering area (n=288) compared to 1.64 $\log_{10}$ cfu/50 cm^2 in the food serving area (n=289). In both our study and theirs, the relatively lower counts found in the diapering area may be attributed to the heavy use of sanitizers in this location because childcare workers are more aware of its potential to serve as a reservoir of enteric pathogens.

According to American Academy of Pediatrics (2011B), childcare facilities can be further classified according to facility type. Home-based childcare usually includes one to twelve children in the home of a caregiver, while a childcare center enrolls a higher number of children in a non-residential setting. Interestingly, after reviewing 12 published microbiological surveys conducted in childcare settings, we found that none of these studies provided a formal comparison of microbial loads found on surfaces or hands in homes versus childcare centers. When this sort of comparison was done using our data, we found no statistically significant difference in APC values when comparing childcare homes and

centers, regardless of the sample type (Table 2.2). However coliform counts associated with hands (p=0.0012), irregular surfaces (p<0.0001) and regular surface samples (p=0.0031) were significantly higher in home-based childcare settings. The fact that APCs did not differ by center type, while coliform counts did, may illustrate a more greater likelihood of "filth" in the home setting relative to the centers. To our knowledge, this is the first such statistical comparison of microbial indicator levels among surfaces and hands in different classifications of childcare facilities.

None of the samples we tested were positive for bacterial pathogens (*E. coli* O157:H7, *Shigella, Salmonella,* or *Campylobacter*). The absence of pathogenic bacteria in our study may in part be due to their relatively poor environmental persistence, as all of the four tested pathogens (*E. coli* O157:H7, *Salmonella spp, Shigella spp,* and *Campylobacter jejuni*) are Gram negative, hence have poorer environmental persistence relative to Gram positives. Interesting, only one previous study reported finding evidence of bacterial pathogens in childcare settings in the absence of an underlying outbreak. Specifically, Staskel et al (2007) collected swab samples from food contact surfaces (such as countertops) and non-food contact surfaces (such as hand-washing sink handles) in 36 Texas childcare centers. Evidence of bacterial contamination was observed for 68 out of the 167 swab samples, as evaluated using a combination of cultural, biochemical, and molecular screening techniques. Further bacterial strain typing yielded 27 different species, with two non-opportunistic pathogens identified: *K. pneumoniae* (2 out of 167 samples) and *Salmonella* paratyphi A (1 out of 167 samples). However, *K. pneumoniae* is mainly responsible for respiratory infections, while *Salmonella* paratyphi can cause enteric fever, but is a disease

rarely encountered in the U.S. (Murray et al., 2005). All said, even though these investigators found potential pathogens in the childcare environment, the public health significance for their findings in remains unknown.

There were 7 presumptively positive results for GI NoV (3 hand samples, 4 environmental samples, n=326) and 41 presumptively positive results for GII NoV (8 hand samples, 33 environmental samples, n=326) (Table 2.3). There were no major differences in NoV prevalence by sample location and childcare facility type (Tables 2.4 and 2.5). Not unexpectedly, the vast majority of childcare classrooms showed no incidence of NoV contamination (Table 2.6).

When dot blot hybridization was used to confirm sample testing presumptively positive for NoV, only one staff hand sample and one refrigerator door handle sample could be definitively confirmed of genogroups I. Even these hybridization signals were extremely weak, suggesting a very low level of contamination. Taken together, these data demonstrate a low prevalence of NoV contamination, suggesting that the childcare locations sampled in this study probably instituted good cleaning and sanitation practices. Previous studies have reported relatively high prevalence of viral pathogen contamination, as high as 50% for influenza A (Boone and Gerba, 2005), and 20-25% for rotavirus (Wilde et al., 1992; Butz et al., 1992). However, these studies were done in conjunction with prior disease outbreaks, so high positivity rates would perhaps have been expected.

There are limitations to our study. First, childcare facility participation was voluntary and hence selection bias was possible, This may have skewed the data collection toward facilities with greater compliance with recommended best hygiene practices; this sort of bias

has been reported by others (Staskel et al., 2007). Secondly, staff received advance notice of our visits, and hence could have made changes in routine disinfection practices prior to sampling. One way to circumvent this problem would have been to take the approach of Cosby et al. (2008), who made multiple visits (e.g. before and after food preparation) to the same site in an effort to determine if microbial load changed as a function of time. Finally due to consent issues, we did not take any samples from the children, who might have served as the most important reservoir for enteric disease agents. Clearly, person-to-person contact has been shown to be the most common transmission route for enteric pathogens the childcare environment, responsible for 43% of outbreaks associated with bacterial agents, 60% of outbreaks associated with parasitic agents, and 40% of viral outbreaks (Lee and Greig, 2008).

The purpose of this study was to characterize microbiological contamination in the childcare environment. In the absence of evidence of pathogen contamination in the environment, our approach of using microbiological indicator data as a proxy allowed us to make some conclusions about the hygiene of the childcare facilities in this study. Overall, our findings of low levels of microbiological indicators and the absence of pathogens support the conclusion that childcare facilities of NC and SC maintained good hygiene practices. Furthermore, observational data on hygiene practices of childcare staff were also collected, and while not discussed here, these will be combined with the microbiological data in an effort to identify potential behavioral risk factors for enteric disease. This information will then be used to inform the development of training materials that can be used by public

health professionals working with childcare staff to assure proper control of the transmission of enteric disease in their facilities.

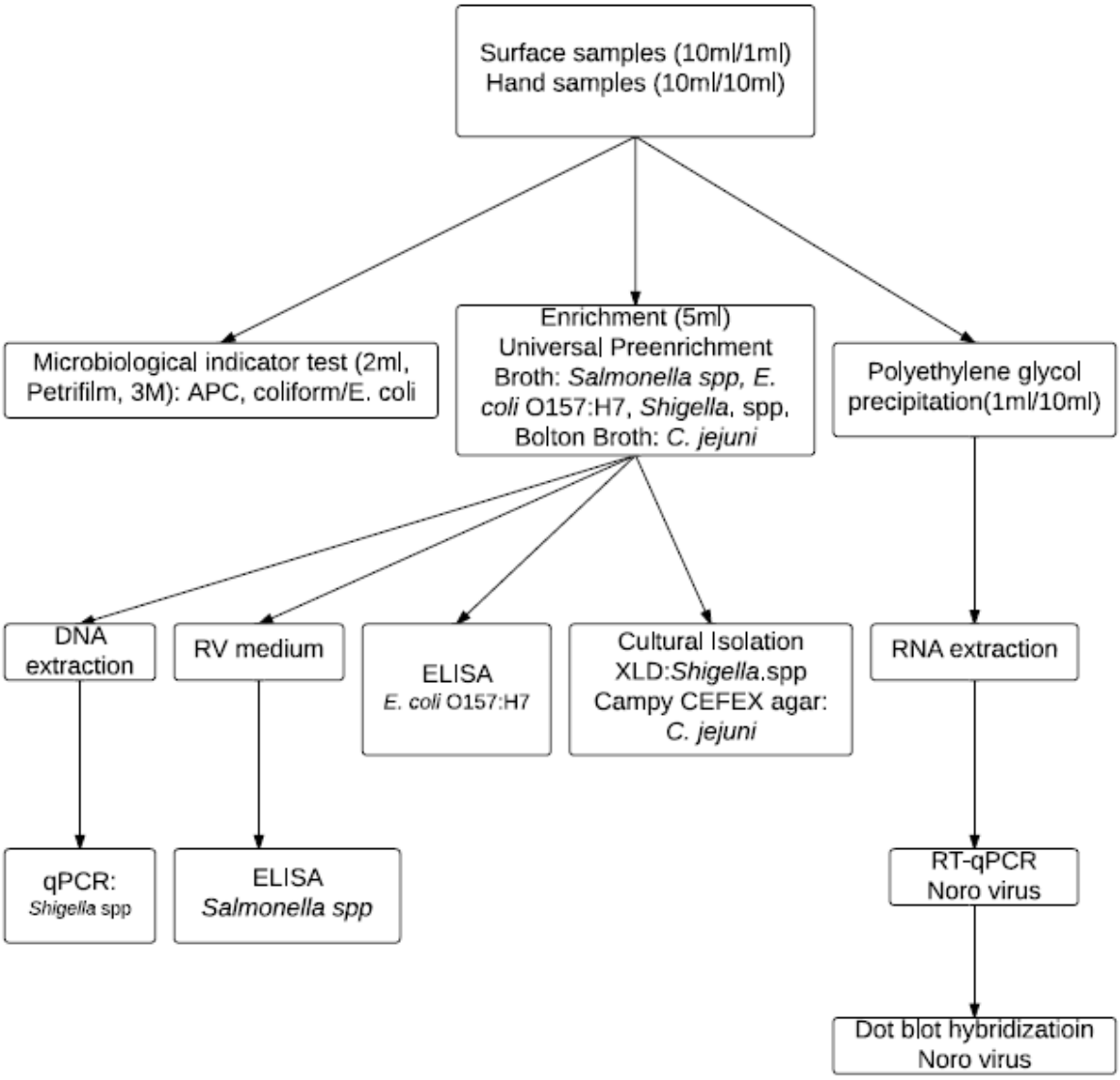
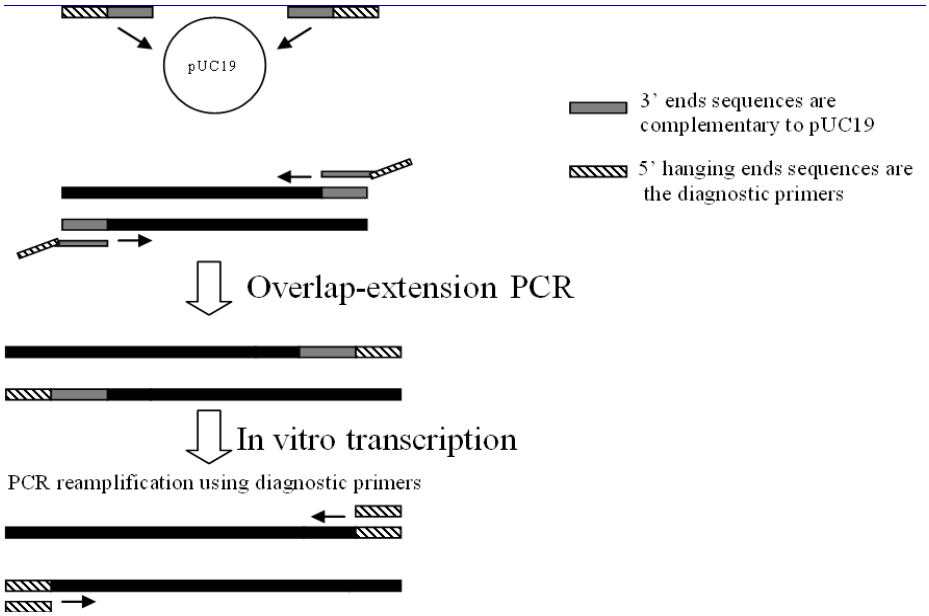


Figure 2.1 Flow diagram of microbiological analysis of environmental samples collected in this study



Primer sequence for overlap extension PCR (Integrated DNA Technologies, Coralville, IA) were

Forward:

GI: 5'*TAATACGACTCACTATAGGG* **CGYTGGATGCGNTTYCATGA** TTC TCA TAG CTC ACG CTG TAG G3'

GII: 5'*TAATACGACTCACTATAGGG***CAAGAGTCAATGTTTAGGTGGATGAG** TTC TCA TAG CTC ACG CTG TAG G3'

Reverse:

GI: 5'**CTTAGACGCCATCATCATTYAC** TCG CTC TGC TAA TCC TGT TAC C3'

GII: 5'**TCGACGCCATCTTCATTCACA** TCG CTC TGC TAA TCC TGT TAC C3'

The commercial plasmid pUC19 (invitrogen, Carlsbad, CA) is used as a template and amplified to introduce diagnostic primer sites (**Bold**) as 5'over-hanging ends of the above set of primers. The T7 RNA polymerase promoter region was included at the 5' end of the forward primer (*italics*) to allow for *in vitro* transcription of the amplicon. The 3' ends of the primers were complementary to the predetermined (pUC19) DNA sequence (underlined). Overlap extension PCR conditions: 30 cycles of 94°C for 30s, 66°C for 30s, 72°C for 30s.

Figure 2.2 Scheme of the construction of IAC according to Abdulmawjood, A. *et al.*, (2002)

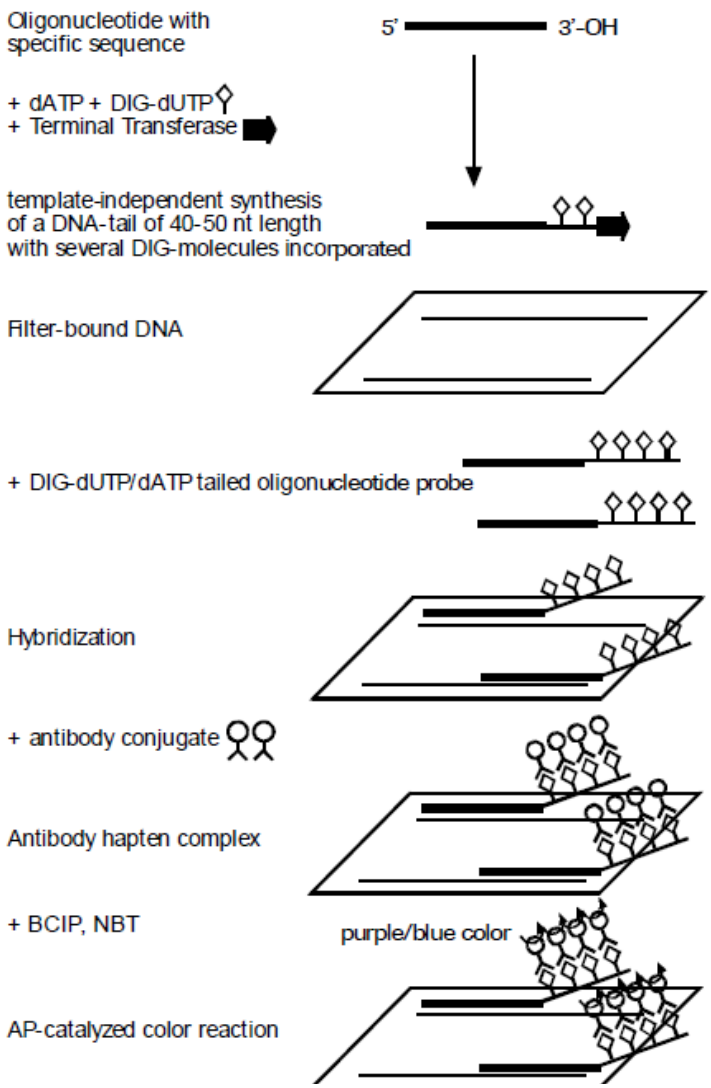


Figure 2.3 Schematic of dot blot hybridization protocol (adapted from Dot blot hybridization manual, Roche Applied science manual, Mannheim, Germany)

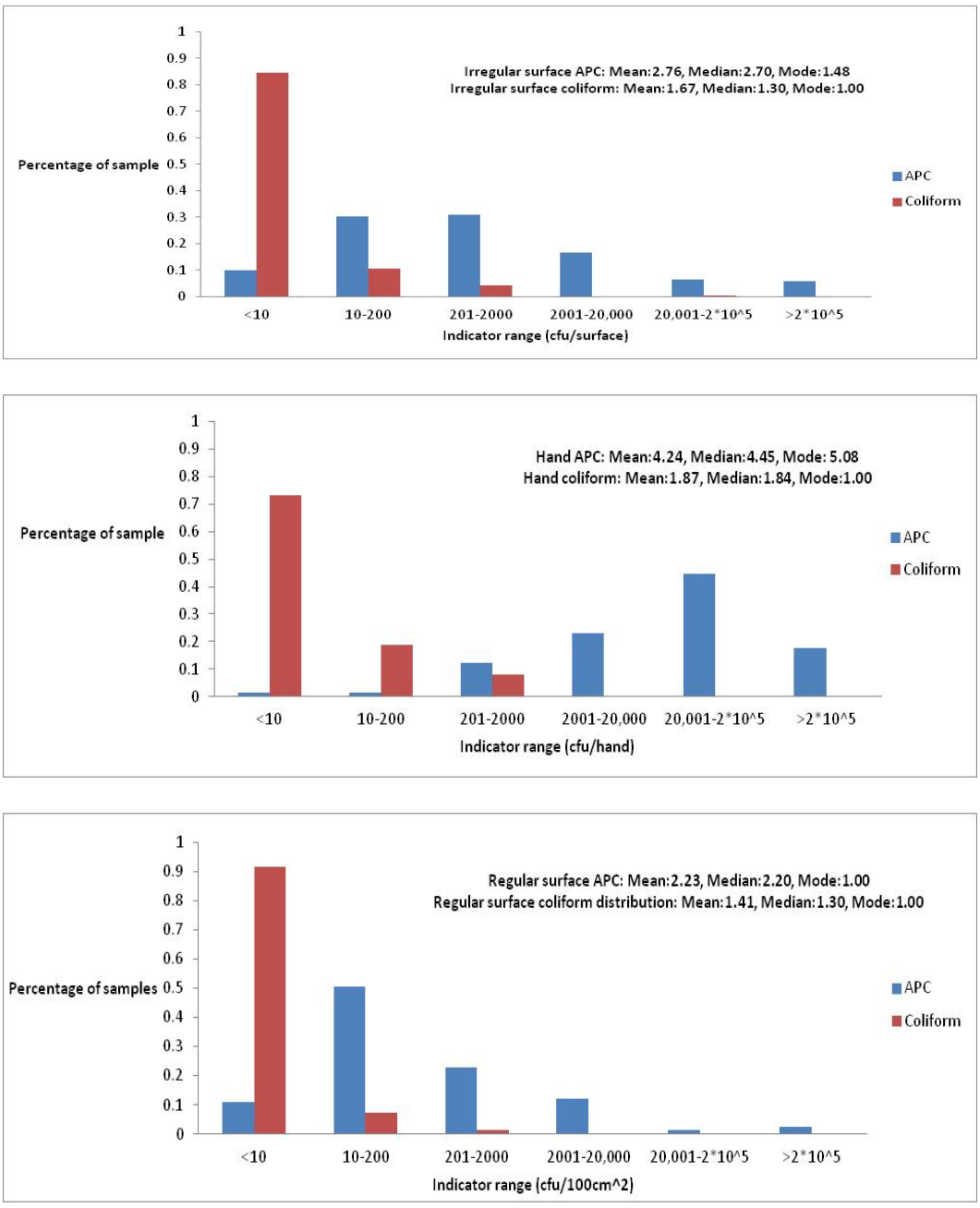


Figure 2.4 Frequency histograms of aerobic plate count (APC) and coliform data corresponding to irregular surfaces, hands and regular surfaces collected from childcare facilities in North and South Carolina. The log mean, median and mode were calculated using samples in the countable range (10-2*10^5 CFU/hand or surface for APC; 10-2*10^{4}cfu/hand or surface for coliform)

Table 2.1 Oligonucleotide primers and fluorescently-labeled probes used in the real time PCR

Target Organism, Target Gene, and Citation	**Primer/Probe Sequences**
Genogroup I noroviruses (ORF-1-ORF2) Kageyama et al., 2003	Primer COG1Forward primer: 5'-CGYTGGATGCGNTTYCATGA-3' Primer COG1Reverse primer: 5'-CTTAGACGCCATCATCATTYAC-3' Probe RING1(a)-TP probe: FAM-5'-AGATYGCGATCYCCTGTCCA-3'-BHQ RING1(b)-TP probe: FAM-5'-AGATCGCGGTCTCCTGTCCA-3'-BHQ
Genogroup II noroviruses (ORF-1-ORF2) Jothikumar et al., 2005	JJV2Forward primer: 5'-CAAGAGTCAATGTTTAGGTGGATGAG-3' COG2Reverse primer: 5'TCGACGCCATCTTCATTCA-3' RING2-TP probe: FAM-5'-TGGGAGGGCGATCGCAATCT-3'-BHQ
Shigella flexnerii (*ipaH* gene) Wang et al., 2007	Shig-1 primer: 5'-CTTGACCGCCTTTCCGATA-3' Shig-2 primer: 5'-AGCGAAAGACTGCTGTCGAAG-3' *Shigella*-probe: 5'-FAM-AACAGGTCGCTGCATGGCTGGAA-BHQ-3'

Table 2.2 Statistical comparison of indicator counts (APC and coliforms) on hands and surfaces collected from North and South Carolina childcare facilities as a function of facility type, i.e., home vs. center

	APC	**COLIFORM**
Stuff hand	P=0.5052 (NS)*	P=0.0012 (S),** homes have higher counts
Irregular surface	P=0.8708 (NS)*	P<0.0001 (S)**, homes have higher counts
Regular surface	P=0.5718 (NS)*	P=0.0031 (S)**, homes have higher counts

* NS, not significant, $p>0.05$

**S, significant, $p<=0.05$

Table 2.3 Prevalence of presumptively positive human NoV contamination in environmental swabs collected from childcare facilities in North and South Carolina by sample type

Sample Type	**Presumptively Positive Samples # positive/total sample # (%)**
Stuff hand sample	10/74 (13.5%)
Irregular surface	23/169 (13.6%)
Regular surface	14/83 (16.9%)

Table 2.4 Prevalence of presumptively positive human NoV contamination in environmental swabs collected from childcare facilities in North and South Carolina by sample location

Sample Location	**Presumptively Positive Samples # positive/total sample # (%)**
Diaper changing area	8/110 (7.3%)
Hard surface toys	10/116(8.6%)
Hand washing faucet handles	8/112 (7.2%)
Refrigerator door handles	7/120 (5.8%)
Eating tables	1/9 (11.1%)
Classroom door knobs	1/6 (16.7%)

Table 2.5 Prevalence of presumptively positive human NoV contamination in environmental swabs collected from childcare facilities in North and South Carolina by facility type

Facility Type	**Presumptively Positive Samples # positive/total sample # (%)**
# of childcare homes with presumptive positive samples	3/9(33.3%)
# of childcare centers with presumptive positive samples	13/31 (41.9%)

Table 2.6 Prevalence of presumptively positive human NoV contamination in environmental swabs collected from childcare facilities in North and South Carolina by classroom type

Classroom Type	**Presumptively Positive Samples # positive/total sample # (%)**
# of classroom with NONE presumptive positive environmental sample	44/62 (71.0%)
# of classroom with ONE presumptive positive environmental sample	9/62 (14.5%)
# of classroom with >ONE presumptive positive environmental samples	9/62 (14.5%)

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CHAPTER 3 An Integrated Microbial and Observational Study of Hygienic Conditions in Childcare Facilities in North and South Carolina

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ABSTRACT

Childcare facilities play an important part for the working family in US nowadays, yet the disease burden caused by enteric pathogens and the associated financial loss make child-care attendance become a concern for the health of children. In this study, observational and microbial investigations were carried out to depict the hygienic conditions and the practices existing within the child-care facilities. Statistical analysis was utilized to associate the results from observational and microbial studies to reveal the risk factors for enteric disease transmission and the corresponding control measures. The prevalence of sanitation training, either for new staff or veteran, is almost two times higher in childcare centers than homes setting, which also have significantly higher level of filth, indicated by coliform level, than centers ($p<0.01$). Better equipment for diaper trash disposal can lessen the level of bacterial load on the stuff hand ($p<0.05$). Well formulated and presented procedures for food preparation and surface cleaning can effectively educate the staff the importance of sanitary practices and check the bacteria, including filth, from contaminating the environment. However, the presence rate of food preparation policy or written procedure

(45%) is almost two times lower than other provisions, e.g surface washing (80%), sick children (97.5%), in childcare facilities. Future studies with larger observation and sampling scale are required for better testing the effectiveness of identified control measures and intervention strategy as well as the commonness of the demographical characteristics of child-care facilities in NC and SC.

INTRODUCTION

Reliance on out-of-home child care in the U.S. has increased dramatically since the 1940s. During WWII only 8.6% of mothers with children under 18 years old were in the work force. Today, 67% of mothers with children under the age of six years work outside the home. Clearly, there has been an associated increase in the demand for organized child care options. It was estimated that nearly 11 million children under the age of five years are in some type of child-care arrangement every week. Of these, 26% are enrolled in center-based arrangements and 6% are in family daycare homes (Child Care Aware of America, 2012).

The close and frequent personal contact between children in child-care settings provides many opportunities for the spread of pathogens, particularly those that cause enteric diseases. In their comprehensive review of English-language journals and public health records, published between 1996 and 2006, Lee and Greig (2008) identified 75 reported enteric outbreaks representing a total of 946 confirmed cases that occurred in child-care settings. More than 93% of identified outbreaks were attributed equally to bacteria and viruses, whereas, viral agents were responsible for more (55.7%) cases. Only 6.6% of

reported outbreaks were attributed to parasitic protozoa. These episodes of GI illness cost the U.S. economy up to $2.3 billion each year, making diarrheal illness not only a public health problem but an economic problem as well (Snowdon et al., 2002).

Enrollment in child care has also been documented to be a risk factor for certain infectious diseases, including those of the gastrointestinal tract (Jones et al, 2006; Lu et al, 2004). Many outbreaks in the day-care center are undoubtedly related to child-to-child transmission of the infecting microorganism. However, transmission related to the contamination of environmental surfaces may also play an important role in these outbreaks, and this warrants further study. Of particular interest are the role of contaminated hands and surfaces with respect to the spread of enteric pathogens.

When studying the role of environmental contamination as a risk factor for enteric diseases, two major approaches have been reported in the literature. In the first, investigators sought to determine the presence and amount of microbial contamination on the hands of child-care workers, and/or on common, high-touch non-porous surfaces, such as diaper-changing tables and toys. Most of these studies used classic microbiological indicators as proxies for general cleanliness (e.g., aerobic plate count and coliforms) or adequate hygienic conditions (e.g., fecal coliforms, generic *E. coli*) (Ekanem et al., 1983; Weniger et al., 1983; Van et al., 1990; LaBorde et al., 1993). In only a few studies did the investigators seek to determine if enteric pathogens were in the environment, and most of these focused on the

presence of viruses as evaluated using molecular techniques (Wilde et al., 1992; Boone and Gerba, 2005).

Observational studies have been the second major approach used to study risk factors for GI illness in the child-care environment. Observational studies are usually designed to evaluate the degree of compliance with regulatory provisions (Chapman et al., 1995; Copeland et al., 2006; Enke et al., 2007; Taylor et al., 2008) or the efficacy of candidate hygiene interventions (e.g. hand washing, diaper changing, food preparation) (Kotch et al., 1994; Sandora et al., 2005; Kotch et al., 2007). In those studies, data were collected by direct observations (Kotch et al., 1994), surveys (Chapman et al., 1995; Copeland et al., 2006; Enke et al., 2007), interviews with staff (Kotch et al., 1994; Sandora et al., 2005; Kotch et al., 2007), or by focus groups (Taylor et al., 2008).

Both microbiological and observational study designs possess their own advantages and disadvantages. It is generally recognized that alone, neither is very effective in establishing causal relationships for GI illness transmission. Aside from one study by Laborde et al. (1993), who used the fecal coliform index as a predictor for the occurrence of diarrheal illness in child-care centers, microbiological and observational study designs are rarely done in conjunction with one another, nor are the data analyzed to determine if microbiological data are predictive of disease risk, human behavior, and/or adherence to recommended practices or procedures. Clearly, a research study integrating both approaches

would maximize the utility of the findings, enabling us to better understand potential risk factors.

As part of a broader project, the aim of which was to identify risk factors associated with potential sources of enteric infection in the child-care environment, the objective of this study was to use statistical analysis to determine potential associations between microbiological (indicator) and observational (survey and audit) data collected from a convenience sample of 31 child-care centers and 9 family daycare homes located in North Carolina and South Carolina. Specifically, we sought to determine if there were significant relationships between the concentrations of key microbial indicators (total aerobic bacteria and coliform counts) and various child-care facility characteristics, practices, and written procedures.

MATERIALS and METHODS

Sampling sites and surface selection

Eighteen (18) North Carolina and 22 South Carolina child-care facilities were visited from September 2010 through February 2011, of which 31 (77.5%) were classified as centers and 9 (22.5%) as homes. Environmental samples were collected from common surfaces (e.g. faucet and refrigerator handles, toys, diaper-changing areas, and tables) as well as hands of care providers and food workers. Each facility was visited once and the average number of samples taken per visit was 16 (range of 10-20). To maintain confidentiality, all sampling data was coded and the analyst conducting the microbiological testing was blinded to sample identity.

Design and implementation of the survey instruments

Directors of the child-care facilities were asked to complete a brief (10 min) questionnaire, while data collectors visiting each facility conducted an observational audit of facility activities. The questionnaire was adapted from a survey of demographic and food safety training information previously collected from child-care center directors in Texas and Iowa (Enke et al., 2007), and from a survey of restaurant food preparation training and policies previously administered to restaurant managers in six states (Green et al., 2007). As shown in Table 3.1, the questionnaire included the following sections: Training, Facility Policies, Facility Characteristics, and Employee and Child Health. Questions on employee and child health were adapted from health log questions used in a previously published child-care study (St. Sauver et al., 1998).

Data collectors conducted environmental audits in up to two classrooms and the food preparation area in each facility. The audit form was designed to assess the hygienic conditions of the rooms and was based on North Carolina and South Carolina environmental health regulations for child-care centers (SC Department of Social Services, 2006; NC ENR DEH, 2007). During the audit, information was collected on characteristics, practices, and procedures, including but not limited to children-provider ratios, diaper trash can and hand sink practices, and written procedures for cleaning and food preparation. Each audit form consisted of a checklist in which data collectors checked “Yes” for compliance, “No” for deviation, or “NA” for “Not applicable.” Space was also provided for data collectors to record deviations or to provide other comments.

To ensure that survey questions were interpreted as intended and audit forms captured the appropriate information, both were pretested in five child-care facilities before data collection began. Following the pretest, minor changes were made to both instruments to improve readability and enhance understanding of the questions.

Surface and hand sample acquisition procedure

The 10 ml 3M^{TM} Swab-Sampler Letheen Broth (3M^{TM}, St Paul, MN) was used for sampling non-porous surfaces. Flat surfaces like food serving and diaper-changing areas were delineated into 100 cm^2 areas using a 10 cm x 10 cm flexible cardboard template (Weber Scientific, Hamilton, NJ). Irregular surfaces like toys or faucet and refrigerator handles were swabbed over the entire area without the aid of a template. The swab was first pressed on the interior wall of its vial to release excess moisture and swabbed over the target area, reversing direction with each stroke. This procedure was repeated twice using different swabbing directions for each replicate. The swab was then deposited back in the Letheen Broth tube, sealed, and placed on ice packs in an insulated cooler.

Sampling of the hands of child-care staff members (care providers and food workers) was done in accordance with the method of Kampf et al. (2006). Briefly, fingertips of each hand were dipped into a Petri plate (9 cm diameter) containing 10 ml of Tryptic Soy Broth (Thermo Fisher Scientific, Lenexa, KS). Participants were instructed to rub their fingertips gently together for a period of 1 min. The sampling fluid for each hand was then aseptically transferred in its entirety to sterile capped plastic vials and placed on ice packs in an insulated

cooler. At the end of the site visit, all samples (hand and surface) were shipped to North Carolina State University for microbial analysis that was initiated within 24 h (usually 12 to 18 h) after sample collection.

Microbial indicator analysis assays

The 3M™ Petrifilm™ Aerobic Count method (3M™, St Paul, MN) was used in accordance with manufacturer instructions to enumerate total aerobic bacterial load. Incubation was at 35 °C for 48 h. Coliform and generic *E. coli* counts were determined using 3M™ Petrifilm™ *E. coli*/Coliform Count Plates per manufacturer instructions with incubation at 35°C for 24-48 h. On these plates, coliforms appeared as red colonies, while generic *E. coli* were blue in color; both were surrounded by gas bubbles. Results were reported as CFU per hand or surface.

Statistical analysis

Statistical analysis was undertaken to determine the relationship between select child-care facility characteristics, practices, and written procedures and concentrations of total aerobic bacteria or total coliforms. Prior to analysis, 17 questions were identified as interesting or relevant. Eight were excluded from the analysis because one or more of the comparison groups contained <=15% of the total sample size (Table 3.2). The absence of positive results for generic *E. coli* precluded the use of these data in the analysis. Because much of the microbiological data was above or below assay detection limits, the nonparametric Mann-Whitney U test (for inferences comparing two populations) and

Kruskal-Wallis test (for inferences comparing three or more populations) were performed using StatCrunch 5.0 (Integrated Analytics LLC, http://statcrunch.stat.ncsu.edu/). Statistical significance was determined at p=0.05. GraphPhad Instat 3.0 (GraphPad Software, Inc, La Jolla, CA) was used for post-hoc paired comparisons if significant differences were identified from the Kruskal-Wallis test.

RESULTS and DISCUSSION

Child Care Facility Characteristics

Forty-five percent (45%) of child-care centers were classified as for-profit and 45% as non-profit facilities. All family daycare homes were for-profit, independently-owned and operated (Table 3.4). Nearly half of the sites (46%; n=18) reported that they participated in the Child and Adult Care Food Program (CACFP), with most identified as family daycare homes or located in North Carolina. Nearly all (93%) centers provided initial training on sanitation practices to new employees, compared with only 56% of daycare homes, which is not surprising given that most daycare homes reported having only one employee, usually the owner/operator. Similarly, more centers (87%) provided on-going training on sanitation practices to their employees compared with daycare homes (56%). Among all facilities surveyed, most directors reported having policies or written procedures for hand washing (82%), diaper changing (88%), surface washing (80%), and cohorting of sick children (98%). In contrast, less than half of the facilities (45%) had a policy or written procedure for food preparation.

Environmental audits were conducted in 57 classrooms [infant rooms (32%); toddler classrooms (35%); combined infant-toddler classrooms (23%); and classrooms with children aged 3-5 years (11%)] (Table 3.5). The mean provider-child ratio across all classrooms was 1.6:6. In classrooms with hand sinks (n=55), 89% were in compliance with regulatory requirements meaning the sink was equipped with warm water, soap, and an approved drying device. All classrooms with diaper-changing areas had diaper-changing surfaces that were clean and in good repair (n=51). In classrooms with diaper trash cans (n=47), 62% were compliant with the American Academy of Pediatrics guidelines (2011), meaning the trash can was plastic-lined and had a hands-free cover. The American Academy of Pediatrics standards were used to evaluate diaper-changing areas, rather than NC and SC regulations, as we believed the former to be a more stringent measure for this surface at high risk for microbiological contamination.

Microbiological Samples

A total of 652 samples were collected for microbial analysis, including 148 hand rinsates, 166 regular (flat) surfaces (100 cm^2), and 338 irregular surfaces. The number of samples collected was much larger than most published microbial studies conducted in child-care settings (Keswick et al., 1983 [n=25]; Worsfold, 1997 [n = 108]; Anderson et al., 2004 [n = 99]; Clayton and Griffith, 2004 [n = 170]; Redmond et al., 2004 [n = 30]; Staskel et al., 2007 [n=167]; Cosby et al., 2008 [n=1149]). Descriptive statistical parameters (mean, median, mode, and data distribution) for all hands and surface samples are presented in Figure 2.4

Microbial Indicators and Child-Care Characteristics

The first statistical analysis was done to compare the difference in surface microbial indicator numbers [aerobic plate counts (APC) and coliforms] between family daycare homes (n=9) and child-care centers (n=31), using the Mann-Whitney U test (Table 3.6). APC counts were not statistically significantly different for any surface when comparing homes to centers. However, samples collected from homes showed significantly higher coliform counts across all surfaces as compared to childcare centers (staff hands, $p = 0.0012$; irregular surfaces, $p < 0.0001$; regular surfaces, $p = 0.0031$; all non-porous surfaces, $p < 0.0001$).

Many published studies have reported that higher enrollment, as associated with centers, increased the risk of gastrointestinal illness among children (Alexander et al., 1990, Berg et al., 1990, Wald et al., 1991), presumably due to a higher likelihood of contact between more children. Thus, one would assume that higher enrollment would also translate to higher degrees of environmental contamination; this was not observed in this study, however. The nine family daycare homes we visited enrolled fewer children (n=6.7 children/facility) compared to 31 child-care centers (n=74.1 children/facility). It is possible that the homes had less physical space than did the centers (square footage was not assessed during the site visit), inherently placing children in closer proximity to one another. Another possible explanation is that the homes we visited had higher proportions of younger (<36 month) children (75.7%) compared to the centers (43.5%). Younger children are more likely to be in diapers. In one prospective community-based study about enteric disease, it was reported that diarrhea was most common among children younger than 36 months old, with

an attack rate of 2.46 episodes per person-year with the rate decreasing with increasing age (Guerrant et al, 1990). This is supported by the findings of four 12-month population-based surveys performed in FoodNet sites. Specifically, Jones et al. (2007) reported that children aged <5 years experienced significantly higher rates of acute diarrheal illness than other age groups. This could be a cautionary sign that child-care facilities that have a higher proportion of younger children (<5 years) might have an increased risk of enteric disease among children. Last but not least, our survey data reveals that centers have a much higher prevalence of initial and ongoing sanitation training as compared to homes (Table 3.4), suggesting there might be a correlation between such training and degree of hygiene in the childcare setting.

In our second analysis, we explored the effect of age, as it relates to classroom structure and degree of environmental contamination. Child-care centers are generally organized by classrooms designated by age, whereas family daycare homes typically include children from many age groups in one classroom or area of the operation. In our study, we categorized classrooms into four groups: infants (0-12 months), toddlers (12-36 months), combined (0-36 months), and other (3-5 years). When comparing these age-specific classrooms, there were statistically significant differences in coliform counts on staff hands when comparing infant to combined classrooms, with latter having higher counts ($p<0.05$). APC counts on surfaces were higher in toddler classrooms than they were in classrooms have children of 3-5 years of age ($p<0.05$). APCs were also higher on irregular surfaces in toddler classrooms relative to classrooms housing 3-5 year olds ($p<0.01$). One explanation for these

findings is that toddlers are more likely to touch surfaces as a means to develop their senses and basic motor skills. In a study by LaBorde et al. (1993), his research team reported that contamination was more frequent on toddler hands than on staff hands (57 vs. 35 percent), and toddler coliform levels were correlated with those of toys and tables. Hence, the dirty hands of toddlers are more likely to contaminate the surfaces in which they come into contact.

Because classroom type can be confounded by provider-to-child ratios, we further refined our analysis to evaluate the differences in indicator counts by provider-to-child ratios within each classroom type, with inconsistent results observed. For instance, in combined classrooms (0-36 months), rooms with higher provider-to-child ratios (1:2) had significantly higher coliform counts on irregular surfaces ($p=0.0003$), while rooms with lower provider-to-child ratios (>=1:4) had higher coliform counts on regular surfaces ($p=0.0006$). In infant classrooms, rooms with higher provider-to-child ratios (between 1:3 and 1:2) had significantly higher coliform counts on provider hands than did infant rooms with provider-to-child ratios of less than 1:3 ($p=0.0018$) (Table 3.6). In the case of higher provider-to-child ratios and elevated bacterial counts, this might be expected; each individual provider has more individual child contacts, resulting in increased microbial loads. Higher microbial counts on irregular surfaces, which include hand sink faucet handles, a refrigerator handles, and toys, may be a consequence of not being cleaned and sanitized as often when staffing is lower (i.e., higher provider-to-child ratios). The fact that, on occasion, rooms with lower provider-to-child ratio had elevated microbial counts, is puzzling and warrants further

investigation. In toddler classrooms, there was no statistically significant difference in indicator counts across rooms with different provider-to-child ratios (Table 3.6). Taken together, and despite the minor differences noted, it appears that provider-to-child ratio had minimal impact on the overall degree of hygiene conditions in child care centers. Interestingly, we were unable to find any studies, observational or microbiological, that sought to determine if a correlation existed between provider-to-child ratios or classroom types and the diarrheal illness rates in children cared for in child-care settings.

We also found significantly higher coliform ($p=0.0048$) counts on surfaces of facilities participating in the CACFP (Table 3.6). This was somewhat surprising. The Child and Adult Care Food Program (CACFP), a federal food assistance program administered through states, reaches more than 3.3 million children from low-income families enrolled in child care (USDA, 2012). As part of this program, state agencies approve sponsoring organizations and independent child-care centers to operate the program at the local level by providing training, technical assistance, and monitoring of nutrition, meal preparation, and food safety practices of participating child-care centers and family daycare homes. Because of the level of technical and training assistance provided by sponsoring organizations, we hypothesized that facilities participating in the CACFP were more likely to maintain higher hygienic standards. There are several possible explanations our unexpected findings. To begin with, most homes (77.8%) were participating in the CACFP and overall coliform counts were higher in homes (Table 3.6). Secondly, as has been widely reported in the literature, training is not always a good proxy for implementation of recommended practices,

including hygiene practices. So just because training is available and offered does not necessarily mean that is transferred into practice. Kotch et al (1994) conducted research on the effectiveness of a curriculum for childcare staff hygienic practices, finding that even after the training, child diarrheal rates remained the same. Furthermore, facilities participating in the CACFP might be located in lower-income neighborhoods where facilities might be older. Older facilities are more likely to have more surfaces that are difficult to clean and sanitize. We did not collect data about the age of the facility or equipment during the audits so cannot confirm this.

We also compared the microbial load between diapering surfaces and food preparation surfaces and found no statistically significant differences ($p>0.05$). Findings similar to ours were also been reported by Cosby et al. (2008), who found no statistically significant difference in APC when comparing these two areas (diaper area: 1.58 $\log_{10}$ cfu/50 cm^2, food area: 1.64 $\log_{10}$ cfu/50 cm^2). This was not surprising because in all facilities visited, food preparation was not conducted in classrooms. Secondly, in all except the family daycare homes, care providers were not responsible for food preparation. Furthermore, the relatively high degree of cleanliness in the diapering (and food preparation) areas may well be due the consistent use of sanitizers, presumably because both North Carolina and South Carolina regulations require diaper stations to be sanitized after each use (Table 3.3).

Facilities that did not have a hands-free, plastic-lined diaper trash can had significantly higher APCs on staff hands ($p=0.0499$). This was not surprising and is

supported by work conducted by Kotch et al (2007). These investigators showed that installing specialized equipment for diaper changing (e.g. foot-activated, roll-out waste bins for diaper disposal to minimize the soiled hand contact with the environment) reduced diarrheal disease rates, presumably because hands were less likely to come in contact with fecal matter and other contaminants. However, to the contrary, in our analysis facilities that had a hands-free, plastic-lined diaper trash can had significantly higher coliform counts on regular surfaces ($p=0.0051$). We can find no plausible explanation for this observation.

As expected, facilities without a written surface cleaning or food preparation policy had significantly higher APC and coliform counts on surface samples (Table 3.6). We could not find any published study that compared the presence and use of such written policies to enteric disease risk or microbiological indicator levels. However, we believe that having a written policy in place is more likely to translate into practice, as this provides workers with a clear guideline regarding how to execute recommended practices. In fact, the written food preparation policy was not as widely implemented as other practices in our visited facilities (Table 3.4), implying the need of enlarging its application in childcare settings.

Limitations

There are several limitations in this study. First, data collected from the facility director was mainly derived through self-report, which may be subject to recall bias (Worsfold, 1997). Secondly, the audit aspects of the study could be confounded by reactivity bias (Bernard, 2000). In an ideal world, we should have made unannounced visits to the

facilities, removing the opportunity for last minute cleaning. Alternatively, it might have been possible to make multiple visits to a single facility over the course of the study, which can further reveal the dynamics of microbial contamination over different times of the day or different days of the year (Cosby et al., 2008). Unfortunately, study designs such as these are more expensive and would have limited our ability to recruit as many child-care facilities as we actually did for this study. Also, it would have been desirable to collect hand rinses from children attending the child-care facilities similar to the study conducted by Laborde et al., (2007), but IRB approval restrictions rendered this infeasible. Thirdly, detection of rotavirus, which is the etiological agent with highest outbreak attack rate (71-100%) among children younger than 2 years in child-care center (Pickering et al., 1981, cited in Guerrant et al, 1990), was omitted due to budget constraints. Finally, participation in the study was voluntary, leading to potential selection bias that could have favored participation by high performing child-care facilities to the exclusion of those having less stringent compliance with recognized hygiene and sanitation best practices.

Conclusion

In general, the child-care facilities visited in this study maintained a high degree of environmental sanitation. Overall, coliform levels (indicators of filth) were higher in family daycare homes versus child-care centers, suggesting a higher degree of hygienic practices in centers compared with homes. This result is further corroborated by the fact that the provision of training to childcare staff on sanitation practices is much higher in centers than home. Diaper-changing areas and food preparation areas were quite clean as indicated by

low APC and coliform counts. Classroom type and provider-to-child ratios had minimal association with environmental contamination in the facilities, although combined classrooms did seem to have a slighter higher level of microbial contamination. In general, implementation of written procedures for hygienic practices, and adherence to them, did appear to have a positive impact on overall microbial loads in child-care facilities. In the future, more expansive studies could be done to determine the efficacy of written policies regarding other activities, e.g. diaper changing and the exclusion or cohorting of ill children. If proven to be effective, this less expensive strategy might be introduced on a larger scale in North and South Carolina, since our results reveal that certain written policies (e.g., those involving food preparation) are not prevalent in childcare setting in these states. Furthermore, it would be useful to perform studies to evaluate the hygienic impact of installation of equipment for hand-washing or managing diaper-changing areas. Nonetheless, this unique study, which combined observational and microbiological data, provided revealing information on the relationship between hygiene indicators and sanitary practices in child-care facilities in the southeastern United States.

Table 3.1 Content of Child Care Facility Director Questionnaire

Training
• Initial training on sanitation practices provided to new employees
• On-going training on sanitation practices provided to employees
Facility Policies
• Policy or written procedure for handwashing
• Policy or written procedure for food preparation
• Policy or written procedure for diaper changing
• Policy or written procedure for surface cleaning
• Policy or written procedure for sick children
Facility Characteristics
• Facility type (family daycare home or child-care center)
• Business type (for profit-chain, for profit-independently owned and operated, or non-profit)*
• Participation in the Child and Adult Care Food Program (CACFP)
• Number of children enrolled by age group
Employee and Child Health
• Children sick with gastrointestinal illness symptoms within 7 days of facility visit
• Employees sick with gastrointestinal illness symptoms within 7 days of facility visit

* All family daycare homes were classified as for profit-independent.

Table 3.2 Questions for statistical analyses about hygienic conditions in child-care facilities

1. Is there a statistically significant difference in indicator counts on sample types between child-care centers (n=31) and daycare homes (n=9)?
2. Is there a statistically significant difference in indicator counts on sample types between facilities that participate in the CACFP* (n=21) and those that do not participate in CACFP (n=17)?
3. Is there a statistically significant difference in indicator counts on sample types among different room types: infant (<12 months) classrooms (n=18), toddler (12-36 months) classrooms (n=20), combined (0-36 months) classrooms (n=13), other (3-5 years) classrooms (n=6), and kitchen area (n=35)?
4. Is there a statistically significant difference in indicator counts on sample types between classrooms in compliance with diaper trashcan guidelines (n=29) and classrooms not in compliance with diaper trashcan guidelines** (n=18)?
5. Is there a statistically significant difference in indicator counts on sample types between facilities without a written food preparation procedures (n=21) and facilities with a written food preparation procedures (n=18)?
6. Is there a statistically significant difference in indicator counts on sample types between combined classrooms with provider-to-child ratio of <=0.25 (n=8) and combined classrooms with provider-to-child ratio of 0.5 (n=4)?
7. Is there a statistically significant difference in indicator counts on sample types between infant classrooms with a provider-to-child ratio of <0.3 (n=9) and infant classrooms with a provider-to-child ratio of 0.3-0.5 (n=9)?
8. Is there a statistically significant difference in indicator counts on sample types between toddler classrooms with a provider-to-child ratio of <=0.2 (n=9), 0.25-0.3 (n=7) and toddler classrooms with a provider-to-child ratio of >0.3 (n=4)?
9. Is there a statistically significant difference in indicator counts on sample types between facilities without written surface cleaning procedures (n=7) and facilities with written surface cleaning procedures (n=32)?

* CACFP is the Child and Adult Care Food Program.

** Compliance is when a diaper trashcan is plastic-lined and has a hands-free cover.

Table 3.3 Select hygiene regulations by state (SOURCE: The Children's Defense Fund, 2008)

	Childcare Centers		Family Daycare Homes	
	North Carolina	South Carolina	North Carolina	South Carolina
Provider-to-child ratio				
(0-9 months)	1:5	1:6	N/A	N/A
(10-27 months)	1:10	1:10	N/A	N/A
(28-48 months)	1:20	1:18	N/A	N/A
Training				
Prevent spread of communicable diseases	No	Yes	No	No
Care of ill child	No	No	No	No
Hand Hygiene				
Required toileting/handwashing activities	No	No	No	No
Specific when staff must wash hands	Yes	Yes	Yes	No
Required locations/number sinks for staff	No	Yes	Yes	No
Diapering				
Requirements for discarding soiled diapers	No	No	Yes	No
Sanitize diaper station after each use	Yes	Yes	No	No
Exclusive sinks for diaper areas	Yes	Yes	No	No
Wear gloves to change diapers	No	No	No	No

Table 3.4 Characteristics of Participating Child Care Facilities

	Percentage of Facilities				
Characteristics	**All**	**NC**	**SC**	**Center**	**Home**
	(n=40)	(n=18)	(n=22)	(n=31)	(n=9)
Training on Sanitation Practices (%)					
Initial training provided to new employees	85.0	77.8	90.9	93.5	55.6
On-going training provided to employees	80.0	83.3	77.3	87.1	55.6
Has Policy or Written Procedure (%)					
Handwashing	82.5	77.8	86.4	83.9	77.8
Food preparation	45.0	38.9	50.0	48.4	33.3
Diaper changing	87.5	83.3	90.9	87.1	88.9
Surface washing	80.0	77.8	81.8	80.6	77.8
Sick children	97.5	94.4	100.0	96.8	100.0
Facility Characteristics					
Business type (%):					
For profit-chain	2.5	0	4.5	3.2	0
For profit-independently owned and operated	55.0	72.2	40.9	41.9	100.0
Non-profit	35.0	22.2	45.5	45.2	0
No answer	7.5	5.6	9.1	9.7	0
Participate in CACFP (%)	42.5	72.2	18.2	32.3	77.8
Mean Number of Children Enrolled by Age Group:					
< 12 months (infants)	5.1	2.6	7.3	6.4	0.6
12-23 months (toddlers)	8.5	5.0	11.5	10.6	1.4
24-35 months	8.9	4.4	12.8	11.2	1.3
3 to 5 years	27.2	21.2	32.4	34.7	2.1
> 5 years	10.7	8.3	12.8	13.6	1.1
All Children	60.4	41.4	76.8	76.6	6.7
Employee and Child Health (%)					
Children sick with gastrointestinal illness symptoms within 7 days of facility visit[a]	40.0	16.7	59.1	51.6	0.0
Employees sick with gastrointestinal illness symptoms within 7 days of facility visit[b]	10.0	0.0	18.2	12.9	0.0

a Two child care centers did not provide a response to this question.
b Three child care centers did not provide a response to this question.

Table 3.5 Classroom Audit Results

	Number of Classrooms				
Characteristics	**All**	**NC**	**SC**	**Center**	**Home**
	(n=57)	(n=20)	(n=37)	(n=48)	(n=9)
Classroom Type (%):					
Infant	31.6	15.0	40.5	37.5	0
Toddler	35.1	20.0	43.2	41.7	0
Combined	22.8	40.0	13.5	10.4	88.9
Other	10.5	25.0	2.7	10.4	11.1
Mean Provider-child ratio	1.6:6.1	1.6:5.6	1.6:6.1	1.7:6.1	1.1: 4.8
Hand sink compliance (%) (n=55)	89.1	85.0	91.4	91.3	77.8
Changing surfaces clean and in good repair (%) (n=51)	100.0	100.0	100.0	100.0	100.0
Diaper trashcan compliance (%) (n=47)	61.7	69.2	58.8	62.8	50.0

Table 3.6 Statistical comparison between surfaces and facility characteristics.

	Staff Hands			Irregular Surfaces			Regular Surfaces			Total Surfaces		
	n	APC	coliform	n	APC	coliform	n	APC	coliform	n	APC	coliform
All												
Homes (N=9)	10	p=0.5052	P=0.0012	30	p=0.8708	P<0.0001	11	p=0.5718	p=0.0031	41	p=0.4654	p<0.0001
Centers (N=31)	64			13			72			211		
Classroom												
Infant (N=18)	17	p=0.3429 df=4	p=0.0568 df=4	46	p=0.0091 df=4	P=0.4268 df=4	26	P=0.5215 df=4	P=0.3486 df=4	72	P=0.0503 df=4	P=0.3222 df=4
Toddler(N=20)	19			48			31			79		
Combined(N=13)	9			34			15			49		
Other(N=6)	6			18			1			19		
Kitchen(N=35)	23			23			10			33		
Provider-to-child Ratio in combined rooms												
<0.25(N=8)	5	P=0.6580	P=1	18	P=0.3449	P=0.0003	9	P=0.1119	P=0.0006	27	P=0.7822	P=0.2462
0.5(N=4)	3			11			5			16		
Provider-to-child Ratio in infant rooms												
<0.3(N=9)	8	P=0.2450	P=0.0018	22	P=0.6439	P=0.4520	14	P=0.5338	P=0.3159	36	P=0.8789	P=0.5804
0.3-0.5(N=9)	9			24			12			36		
Provider-to-child Ratio in toddler rooms												
<=0.2(N=9)	9	P=0.8812 df=2	P=0.7456 df=2	23	P=0.8093 df=2	P=0.9093 df=2	12	P=0.8186 df=2	P=0.5829 df=2	35	P=0.8815 df=2	P=0.5829 df=2
0.25-0.3(N=7)	6			18			10			28		
>0.3 (N=4)	4			7			9			16		
Written Food Preparation Procedure												
Without (N=21)	41	P=0.2176	P=0.1740	90	P=0.0003	P=0.0240	48	P=0.0491	P=0.4675	138	P=0.0002	P=0.0193
With (N=18)	30			73			32			105		

Table 3.6 Continued

	Staff Hands			Irregular Surfaces			Regular Surfaces			Total Surfaces		
	n	APC	coliform	n	APC	coliform	n	APC	coliform	n	APC	coliform
Participation in CACFP												
No (N=21)	44	P=0.6559	P=0.3697	102	P=0.0743	P=0.6885	49	P=0.3729	P<0.0001	151	P=0.0164	P=0.0048
Yes (N=17)	27			61			33			94		
Hands-free, Plastic-Lined Diaper Trashcan												
Without (N=18)	31	P=0.0499	P=0.6528	41	P=0.4973	P=0.4385	27	P=0.8448	P=0.0051	68	P=0.8402	P=0.3113
With (N=29)	31			93			49			142		
Written Surface Cleaning Procedure												
Without (N=7)	15	P=0.9493	P=0.2861	32	P=0.0017	P=0.0470	17	P=0.1829	P=0.0434	49	P=0.0014	P=0.0126
With (N=32)	56			131			63			194		

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CHAPTER 4 Conclusions

Reliance on out-of-home child-care in the U.S. has increased dramatically since the 1940s. Today, 67% of mothers with children under the age of six years work outside the home. It is estimated that nearly 11 million children under the age of five years are in some type of child-care arrangement every week. The close and frequent personal contact between children in child-care settings provides many opportunities for the spread of pathogens, particularly those that cause enteric diseases. Episodes of gastrointestinal illness associated with child-care are estimated to cost the U.S. economy over $2 billion each year, making diarrheal illness not only a public health problem but an economic problem as well.

Enrollment in child-care has also been documented to be a risk factor for certain infectious diseases, and although many outbreaks in the day-care environment are undoubtedly related to child-to-child transmission, the role of environmental contamination cannot be overlooked. Of particular interest is the importance of contaminated hands and surfaces with respect to the spread of enteric pathogens. When studying the role of environmental contamination as a risk factor for enteric diseases, two major approaches have been reported in the literature. In the first, investigators seek to determine the presence and amount of microbial contamination on the hands of child-care workers, and/or on common, high-touch non-porous surfaces, such as diaper-changing tables and toys. Observational studies have been the second major approach used to study risk factors for gastrointestinal illness in the child-care environment. These are usually designed to evaluate the degree of compliance with regulatory provisions or the efficacy of candidate hygiene interventions (e.g. hand-washing, diaper changing, food preparation).

Both microbiological and observational study designs possess their own advantages and disadvantages, but it is generally recognized that alone, neither is very effective in establishing causal relationships for illness transmission. Further, microbiological and observational study designs are rarely done in conjunction with one another, nor are the data analyzed to determine if microbiological data are predictive of disease risk, human behavior, and/or adherence to recommended practices or procedures. Clearly, a research study integrating both approaches would maximize the utility of such findings, enabling us to better understand potential risk factors for gastrointestinal disease transmission in this important environment. This was indeed the justification for the approach reported in this dissertation.

This dissertation represents work on a broad project, the purpose of which was to identify risk factors associated with potential sources of enteric infection in the child-care environment. The focus was on analyzing environmental samples for various microbiological indicators, and to use that data to determine if there were statistically significant associations between microbiological load and child-care facility characteristics, practices, and written procedures. The dissertation began with a comprehensive literature review that provided an overview of the previous studies on microbial contamination sources, gastrointestinal disease transmission, control measures and their efficacy, and associated training and regulation. It sets up a sound theoretical foundation for the subsequent research activities. Significant findings from the literature review included the following:

1. Pathogenic *E. coli*, *Salmonella* spp, *Shigella* spp, hepatitis A virus, rotavirus and norovius were the main etiological agents responsible for enteric diseases in children attending child-care facilities.
2. Person-to-person and various environmental surfaces were the main routes for enteric pathogens transmission.
3. Pathogenic bacteria and viruses were rarely detected in environmental samples derived from child-care facilities on a routine basis, but were occasionally detected when a facility had been linked to an outbreak.
4. Education and intervention targeting child-care center staff can be effective strategies to raise awareness regarding good hygiene practices, and potentially mitigate diarrheal disease burden in children.
5. Vaccination, cohorting convalescing children, and environmental sanitation were effective practices to prevent enteric disease transmission in the child-care environment.
6. Child-care staffs training requirements vary by state, but in general, the requirements for child-care centers are more stringent than those for child-care homes.

In the first research study, environmental samples (corresponding to high touch surfaces and the hands of caretakers) were taken from representative child-care facilities located in North and South Carolina. The most significant findings from this study included the following:

1. APC and coliform levels on the hands of staff members were higher than those on common surfaces.

2. Pathogenic bacteria (*E. coli* O157:H7, *Salmonella* spp, *Shigella* spp, *Campylobacter jejuni*) could not be detected on environmental samples derived from the child-care samples analyzed in this study. Evidence of low levels of norovirus contamination was occasionally detectable, but the public health significance of this is unknown.

3. There was no significant difference in APC and coliform counts when comparing the diaper changing and food preparation areas of the child-care centers evaluated in this study.

Taken together, the higher coliform and APC counts on hand samples derived from staff members illustrates that hands may be a good habitat for microorganisms and could serve as a potential contamination source due to tactile events. The absence of pathogenic bacteria on all environmental surfaces suggests that the child-care facilities practiced sufficient cleaning and sanitation. However, it should be noted that Gram negative pathogens (*E. coli* O157:H7, *Salmonella* spp, *Shigella* spp, *Campylobacter jejuni*) have limited environmental persistence, which could also explain their absence in the samples. The conventional notion that diaper changing areas are more prone contamination was refuted in this study, probably because of a high degree of awareness of the potential for such surfaces to be contaminated, leading to increased cleaning and sanitation stringency.

In the second study, statistical analysis was used to determine if there were significant relationships between the microbiological indicator data and certain child-care practices, the latter of which were previously determined by a combination of survey and observation. We found that:

1. Child-care homes had statistically significantly higher coliform counts on environmental samples than did child-care centers.

2. Toddler classrooms had statistically significantly higher APC counts on common surfaces than did classrooms with older children.

3. Coliform counts were statistically significantly higher on the surfaces of facilities participating in the CACFP program.

4. Facilities without written surface washing or food preparation policies had statistically significantly higher APC and coliform counts on surface samples.

The nonparametric statistical analysis described in this section of the dissertation seeks to determine if elevated microbiological indicator levels are associated with key child-care characteristics or practices. In other words, can the indicator levels be predictive of good or bad practices, and/or vice versa. The elevated coliform counts in child-care homes compared to centers were probably due to a combination of younger children and less stringent staff training in sanitation and hygiene in the former. The fact that toddler classrooms had higher surface APCs may be reflective of mobile children in diapers and a developmental stage in which objects are frequently touched. Although theoretically, the CACFP program brings more training into participating child-care facility staff, higher coliform counts in the facilities participating in CACFP might suggest that training on sanitary practices does not always equate to action. On the other hand, the fact that facilities having written policies on food preparation and surface washing had lower surface coliform

and APC values suggests that this sort of sanitation and hygiene intervention might indeed be effective.

There are some limitations to our study design that invariably impact the type and quality of conclusions we can make. For example:

1. Child-care facility participation was voluntary, meaning that the study could have inherently selected for facilities with better sanitation and hygiene practices.

2. Each facility received advanced notice prior to the site visit, allowing them the opportunity to enhance sanitation and hygiene practices and behaviors if so wished, essentially cloaking real behaviors.

3. Children's hands, which are undoubtedly a significant source of enteric pathogen transmission, were not included in the sampling plans.

4. Facility director surveys were conducted by self-report questionnaire, which could have resulted in inaccuracies due to recall bias.

5. The presence of external auditors could have altered the normal activities in the classrooms such that observations were not necessarily representative.

6. Due to financial constraints, we did not screen for rotavirus, even though it is the leading cause of diarrheal disease in children younger than 2 years.

Refinements could address some of these limitations; such refinements might include the following:

1. Inclusion of children's hands in the environmental sampling protocol.

2. Inclusion of rotavirus screening in the environmental sample testing protocols.

3. A study design in which multiple visits were made to a single facility, at different days, times, and/or seasons, which could have helped reduce observation bias and perhaps provided a better representation of the dynamics of microbiological indicator loads over time.

4. Visiting child-care facilities without advance notice would have eliminated the opportunity for advance site preparation, perhaps yielding results more representative of real-world conditions and behaviors.

5. Non-microbial indicators could have been used to supplement or even substitute the microbiological indicators used in this study. Candidate methods include ATP bioluminescence (an indicator for filth); hemoglobin (a marker for blood); amylase (an indicator for urine, saliva and sweat); and/or protein (an indicator for general hygiene).

This study begs one to answer the question "what is cleanliness?" Although there are a number of different definitions floating about, the term "clean" generally means freedom from dirt, marks, stains, pollution, foreign matter, impurities, etc. This definition is, inherently, subjective. What is "clean" for one person may not be "clean" for another. Within the confines of technical definitions of clean, the child-care environments we sampled were, in large part, free of indicators of fecal contamination and microbiological pathogens. Varying levels of non-pathogenic microbes continued to persist on surfaces and hands, but

this is to be expected, as no surface in the natural environment would be expected to be microbe-free.

Overall, our findings of low levels of microbiological indicators and the absence of pathogens in environmental samples and hands support the conclusion that the general hygienic level in child-care facilities of North and South Carolina was quite good. In the absence of evidence of pathogen contamination in the environment, our approach of using microbiological indicator data as a proxy allowed us to make some conclusions about the overall hygiene of the childcare facilities in this study. Despite potential limitations, this unique study, which combined observational and microbiological data, provided revealing information on the relationship between hygiene indicators and sanitary practices in child-care facilities in the southeastern United States. It may ultimately serve as a model for similar types of studies in the future.

APPENDIX

Validation of Molecular-Based Methods Used for Pathogen Detection

Introduction

Bacterial (e.g. *E. coli* O157:H7, *Shigella spp*, *Salmonella*) and viral (norovirus, rotavirus) agents are the major causes of enteric disease in children attending childcare facilities (Lee and Grieg, 2008). With the advent of molecular techniques, methods like PCR can be used as a complement to standard cultural procedures for enteric bacteria detection, saving time and money. Genus, species, and/or strain-specific genes have been identified for key pathogens, e.g., the *eae* gene that encodes the intimin protein of *E. coli* O157:H7 (Sharma, 2006); the *ipaH* gene that encodes the invasion plasmid antigen H in *Shigella spp*. (Wang, et al., 2007); and the *invA* gene which encodes proteins involved in flagellar biosynthesis or the secretion of virulence determinants for *Salmonella spp*. (Ginocchio and Galan, 1995). These are all important virulence determinants with specificity for these key foodborne pathogens. Noroviruses, which tend to be quite genetically diverse, can be discriminated at the genogroup level by applying RT-qPCR methods targeting the ORF1-ORF2 junction (Kageyama et al., 2003; Jothikumar et al., 2005). In initial studies prior to receipt of naturally contaminated environmental samples derived from childcare environments, we sought to investigate the utility of using a combination of cultural enrichment and molecular-based assays for pathogen detection. This appendix describes the results of these studies.

Materials and Methods

Bacterial and viral strains.

E. coli O157:H7 ATCC43895 and *Shigella. flexneri* ATCC 12022 were obtained from American Type Culture Collection (Rockville, MD). A *Salmonella enterica* serovar. Typhimurium strain, which was isolated from an infected human and naturally resistant to ampicillin (courtesy of Dr. W. Gebreyes, Department of Veterinary Preventive Medicine, The Ohio State University, Columbus, OH), was also used in this study. The bacterial stocks were stored in glycerol at -80^{0}C prior to resuscitation. Working liquid cultures were obtained by growing bacteria in Brain Heart Infusion (BHI; OXOID Ltd., Basingstoke, U.K.) broth for 16 h at 37 ^{0}C. Enumeration of bacterial stock culture titers was done by performing 10-fold serial dilutions of each target bacterium prepared in 0.1% (wt/vol) sterile peptone water (Becton Dickinson, Sparks, MD). One-hundred µl of each dilution was spread plated on agar-solidified BHI broth, and colonies were counted after incubation at 37 ^{0}C for 24 h.

Representative human norovirus GI (Norwalk virus) and GII (Snow Mountain virus) were obtained from Dr. C.L. Moe (Emory University, Atlanta, GA) as fecal specimens collected from experimentally infected volunteers. The specimens were suspended 20% in phosphate buffered saline (PBS), aliquoted and stored at -80°C until use.

qPCR standard curves for bacterial pathogens.

One ml aliquots of overnight cultures of each bacterium were heated at 100^{0}C for 5 min in a water bath to release DNA, prior to flash cooling on ice. Ten-fold serial dilutions of

these suspensions were made and used directly in qPCR assays to construct a semi-quantitative standard curve for each bacterium. All experiments were repeated in duplicate.

Reverse transcription (RT)-qPCR standard curves for human NoV.

To obtain a standard curve for both GI and GII human NoV, fecal stocks were serially diluted, extracted for RNA isolation. And the template RNA used in RT-qPCR. All experiments were repeated in duplicate.

Amplification and detection of target bacteria using qPCR.

Target genes, primers, and Taqman® probe sequences, and amplicon sizes, for the three bacterial qPCR assays (*Salmonella* Typhimurium, *Shigella. flexneri*, and *E. coli* O157:H7) are shown in Table 5.1. The primers and probes were synthesized by Integrated DNA Technologies (Coralville, IA). Each probe was labeled with a reporter dye (FAM) on the 5' end and a quencher dye (BHQ) on the 3' end. Amplifications for *E. coli* O157:H7 and *S. flexneri* were done in 25 µl reaction volumes containing 2 µl extracted DNA, 0.4 µM primers, 0.2 µM probe, 0.2 µM of dNTP mixture (Applied Biosystems, Foster City, CA), 0.5 µl reference dye ROX (Invitrogen, Carlsbad, CA) and 15.55 µl DPEC-treated water added to PCR buffer, $MgCl_2$ and enzyme mixture as recommended by the manufacture of Plantium Taq^{TM} polymerase (Invitrogen). The real time PCR parameters for *S. flexneri* and *E. coli* O157:H7 included an initial cycle of 95^0C for 2 min followed by 40 cycles of 95^0C for 10s, 55^0C for 20s and 72^0C for 20s.

For amplification of *Salmonella*. Typhimurium DNA, the 25 µl reaction mixture contained 2.5 µl DNA, 0.24 µM primers, 0.2 µM probe, 0.4 µM dNTP mixture, 0.5 µl reference dye ROX, and 0.1 mg/ml bovine serum albumin (BSA; Promega, Madison, WI) suspended in 13.55 µl DPEC-treated water to which was added the concentrations of PCR buffer, $MgCl_2$ and enzyme mixture recommended by the manufacturer of Plantium Taq™ polymerase. PCR was carried out using an initial cycle of 94 ^{0}C for 2 min, followed by 40 cycles of 94 ^{0}C for 20s and 60 ^{0}C for 30s.

For all bacterial pathogens, amplifications were done using a StepOne™ Real-Time PCR system (Applied Biosystems) using the StepOne™ softerware V2.0. For all reactions, positive (target bacterial DNA) and negative (DPEC-treated water) were included.

Evaluation of feasibility of using swab sampling in conjunction with qPCR for detection of environmental contamination with enteric bacterial pathogens.

A series of experiments were conducted to determine if it was possible to recover candidate bacterial pathogens from surfaces, enrich them by cultural methods, and directly detect them using qPCR. Briefly, 50 µl 10-fold serial dilutions of bacterial stock solutions were spotted onto small stainless steel surface coupons, delivering inoculum concentrations of 10^1, 10^2, 10^3, and 10^4 CFU/surface. After drying for 30 min, each coupon was swabbed for recovery of the inoculum using the 3M swab sampler suspended in 10 ml of Letheen broth (3M, St Paul, MN). Briefly, the head of swab was rubbed against the interior wall of the sampler vial to remove excess moisture, and then swabbed across the entire stainless steel

surface, reversing the direction at each stroke, and dipped the swab back into the vial. This procedure was repeated twice using different swabbing directions, for a total of three times. The samples were held at 4 ^{0}C for 24 h to mimic sample transportation condition, after which 2.5 ml of each swab suspension was inoculated into 22.5 ml of universal pre-enrichment broth (UPB; Difco Laboratories, Sparks, MD), followed by incubation for 24 h at 37 ^{0}C. UPB inoculated with 2.5 ml sterile Letheen broth was used as a negative control. For isolation of *E. coli* O157:H7 and *S. flexneri*, the UPB was streaked onto sorbitol MacConkey agar (Difco), with colorless colonies representing sorbitol-negative *E. coli* O157:H7, and xylose lysine desoxycholate (XLD) (Difco) agar, with red colonies indicative of *Shigella spp.* For *Salmonella* isolation, 1 ml of the enriched UPB was transferred to 9 ml Rapport-Vassiliadis broth (RV; Becton-Dickinson) and another 1 ml aliquot transferred to 9 ml tetrathionate broth (TT; Difco), followed by incubation for another 24 h at 37 ^{0}C. The RV and TT were then streaked onto xylose lactose tergitol™ 4 (XLT 4) agar plates (Difco) in accordance with the Bacteriological Analytical Manual of FDA (Andrews et al., 2011). Typical *Salmonella* colonies were yellow to red in color with black centers.

In addition to cultural isolation, 300 µl aliquots of overnight-incubated UPB as was extracted for DNA isolation using the MasterPure^{TM} DNA Purification kit (Epicentre Biotechnologies, Madison, WI) according to manufacturer's instructions, using a resuspension volume of 30 µl in nuclease-free sterile water (Fisher Scientific, Fair Lawn, NJ). The genomic DNA was serially diluted in DPEC-treated water prior to amplification

using the qPCR protocols described above. All experiments were repeated in triplicate. A flow diagram of the procedure is provided in Figure 5.1.

Optimization of Internal Amplification Control (IAC) for use in reverse transcription (RT)-qPCR detection of human noroviruses.

The construction of the internal amplification control (IAC), RNA extraction and RT-qPCR conditions for virus detection are described in Chapter 2. In order to determine the optimal concentration of the IAC for co-amplification, concentrated IAC was serially diluted in DPEC-treated nuclease-free sterile water and subject to RT-qPCR amplification. The dilution corresponding to a Ct value of approximately 30 was used in co-amplification assays as applied to detection of NoV in naturally contaminated environmental samples derived from childcare centers.

Results and Discussion

The detection limits of the *E. coli* O157:H7 qPCR method was around 10^2 CFU/reaction (Figure 5.2). When the organisms were artificially inoculated onto stainless steel coupons, swabbed for recovery then extracted for DNA isolation and subjected to qPCR for detection, a slightly improved detection limit was achieved, around 10^1 CFU/swab. Specifically, for the 10^3, 10^2, 10^1, and 10^0 CFU/surface inoculum levels, 3/3 replicates were positive for each 10^1 cfu/surface inoculum and 2/3 were positive for each 10^0 inoculum. The PCR detection limit (10^2 CFU/reaction) was higher (less sensitive) than required by official standard cultural methods, which must be able to detect 1 CFU per sample (Feng, P., 2012; Petridis et al 2002). This result informed our decision to use a standardized, AOAC-

approved Enzyme Linked Immunosorbent Assay (ELISA) for detection of *E. coli* O157:H7 in enriched samples taken from the childcare facilities, as this method has a defined detection limit of 1-25 CFU/25g sample as per the manufacturer (3M).

On the other hand, the qPCR standard curves for *S. flexneri* and *Salmonella* Typhimurium demonstrated a detection limit of 10^0 CFU/reaction (Figure 5.3 and 5.4) and a similar detection limit was observed for the enriched swab samples. Specifically, for the 10^3, 10^2, 10^1, and 10^0 cfu/surface inoculum levels for *Shigella*, 3/3 replicates were positive for all inoculum levels, including the 10^0 cfu/surface concentration. For *Salmonella*, 3/3 replicates were positive at the 10^1 cfu/surface inoculum level, and 2/3 replicates were positive at 10^0 cfu/surface. This is much more in keeping with the detection requirements of standard cultural methods (Lampel, K.A., 2012 , Hammack, T.,2012). Because the ELISA method was chosen for detection of *E. coli* O157:H7, and to facilitate workflow and sample processing speed, a commercial ELISA method (3M) was also chosen for detection of *Salmonella* in naturally contaminated environmental samples. In the absence of a commercial ELISA method, we continued to use the PCR-based detection method for *S. flexneri*.

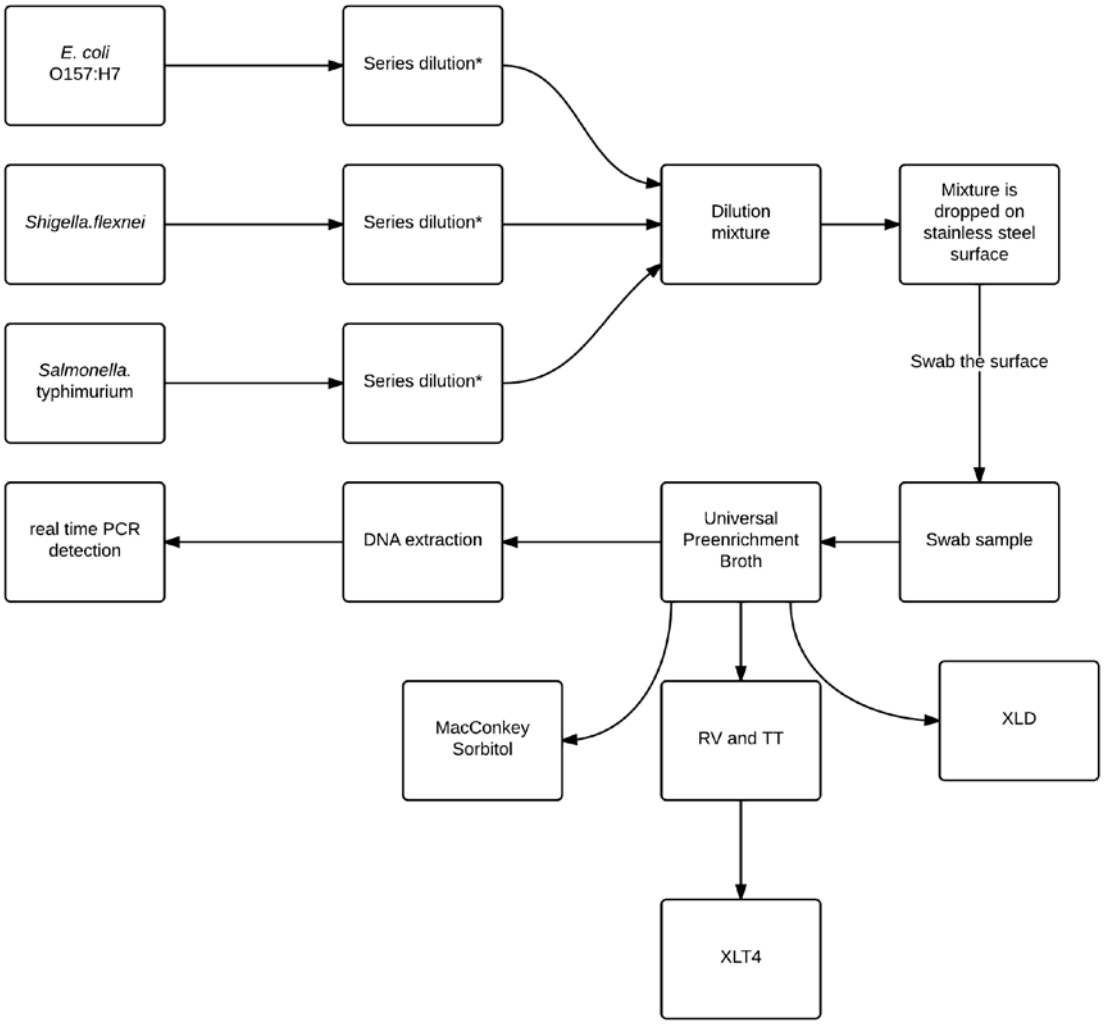
Standard curves for human NoV GI and GII are shown in Figure 5.5 and 5.6; since the virus is not cultivable, detection limits are expressed by dilution series. Specifically, the most dilute sample still providing quantifiable RT-qPCR detection is designated as corresponding to a virus concentration of 1 RT-qPCR amplifiable unit (RT-qPCRU). Based

on these means of estimating stock virus titers, the GII stock contained approx. $0.5*10^6$ RT-qPCRU/µl and the GI strain approximately $0.5*10^2$ RT-qPCRU/µl

Studies were also undertaken to optimize the IAC concentration used in co-amplification reactions. In these experiments, the IAC was 10-fold serially diluted and amplified without target template; the IAC dilution achieving a Ct value around 30 was selected for the co-amplification studies because it would be less likely to interfere with amplification of the target, particularly if the target was at low copy number (Suo et al., 2010). This equated to an IAC concentration of $1.1*10^{-2}$ ng/µl (10^{-4} dilution of IAC stock) for GI NoV and $3.4*10^{-2}$ng/µl (10^{-5} dilution of IAC stock) for GII NoV. Serially diluted human NoV GI and GII were detected by RT-qPCR both with and without inclusion of the IAC. The GI NoV stock could be detected at the 10^{-3} dilution (albeit with a high Ct value of 40) without the IAC, and but only at the 10^{-2} dilution with the IAC. In short, the presence of the IAC increased the detection limit (made the assay less sensitive) by 1 $\log_{10}$ (Table 5.2). Aside from this, the presence of the IAC made little difference in target Ct values. On the other hand, the GII NoV strain could be detected up to a 10^{-6} dilution (high Ct value of 41.4), but only to the 10^{-4} dilution in the presence of the IAC. Effectively, the presence of the IAC increased the detection limit (made the assay less sensitive) by 2 $\log_{10}$ (Table 5.3). Clearly, for both GI and GII NoV assays, the IAC resulted in some degree of reduced amplification efficiency of the target, presumably due to competition. The greater decrease in detection sensitivity for the GII assay relative to the GI assay was probably due to the somewhat higher concentration of IAC used in the former reaction mixture. Based on these results, we

concluded that the presence of the IAC had some impact on assay detection limits, and hence the decision was made to screen naturally contaminated childcare environmental samples for human NoV contamination both with and without inclusion of the IAC.

The sum total of these findings helped in the choice and optimization of the methods that were eventually used in Chapter 2 of this dissertation.



*The diluted cultures were also used for bacterial enumeration and procurement of standard curve of real-time PCR

Figure 5.1 Schematic diagram of experimental design for the methods validation study

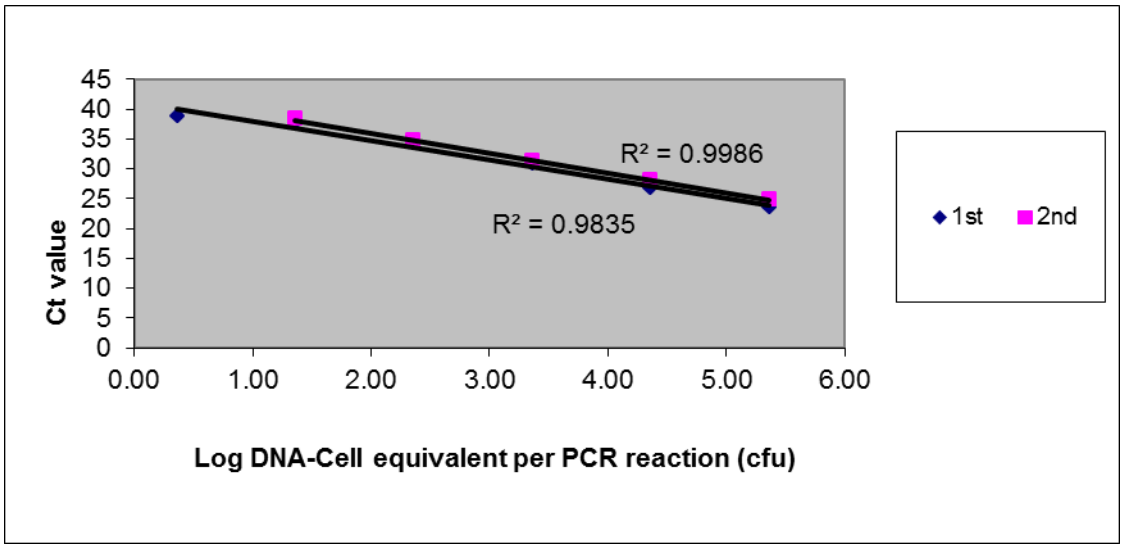


Figure 5.2 qPCR standard curve for *E. coli* O157:H7

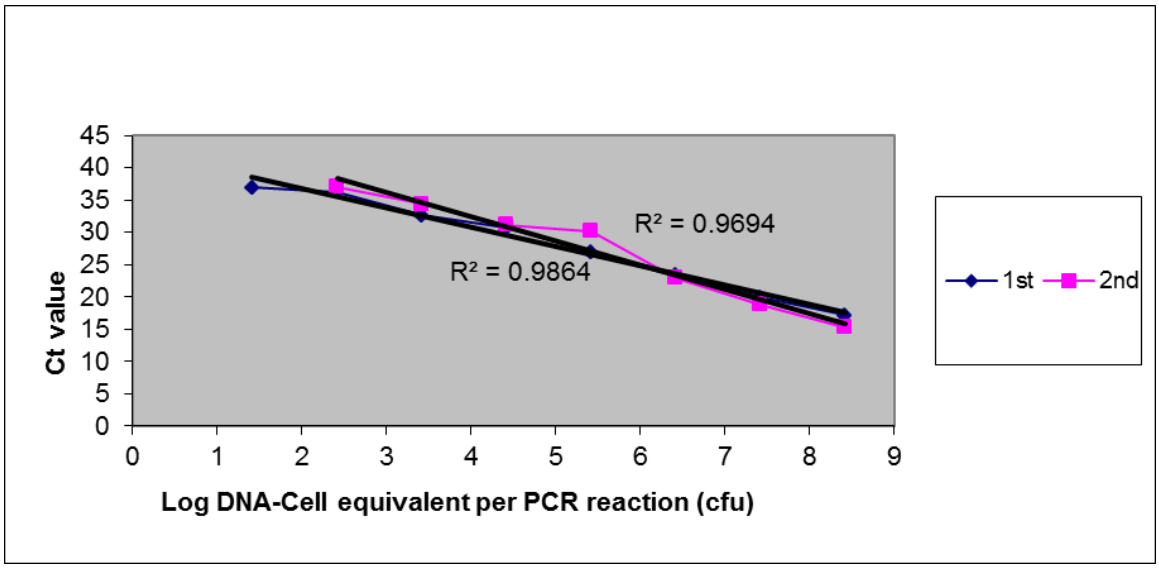


Figure 5.3 qPCR standard curve for *Salmonella* Typhimurium

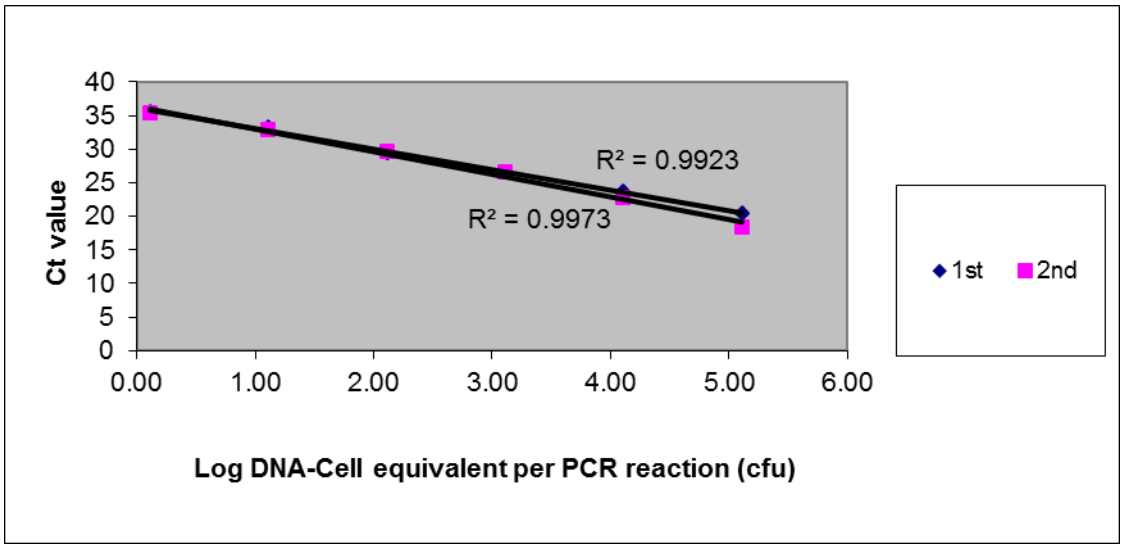


Figure 5.4 qPCR standard curve for *Shigella flexneri*

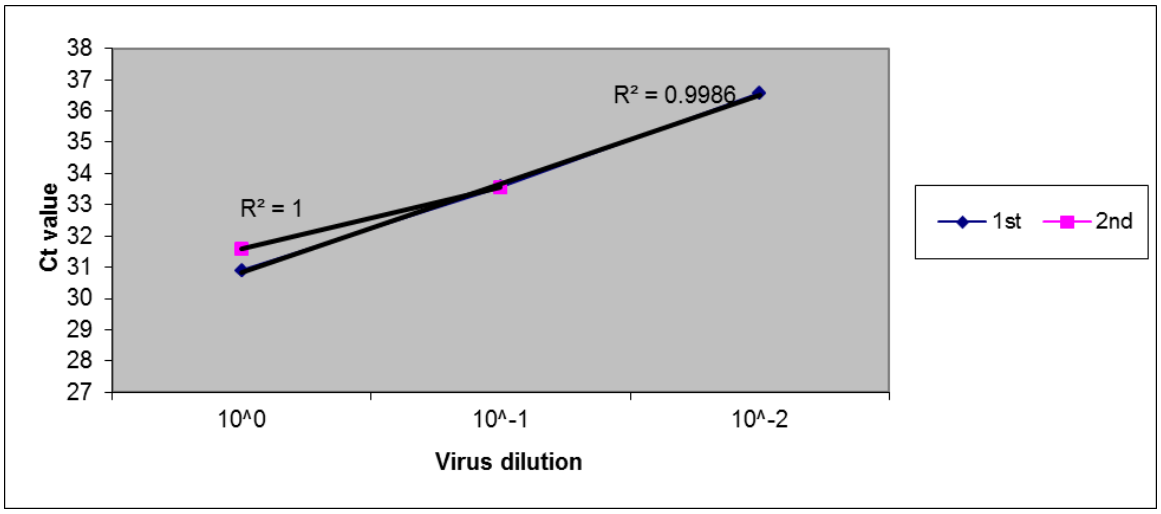


Figure 5.5 RT-qPCR standard curve for human NoV GI

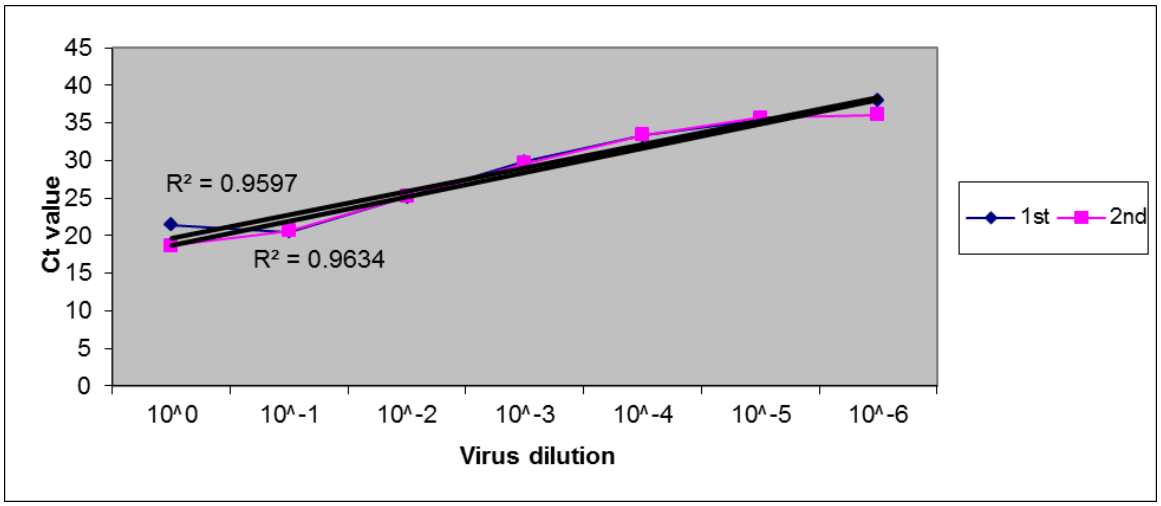


Figure 5.6 RT-qPCR standard curve for human NoV GII

Table 5.1 Oligonucleotide primers and fluorescent probes used in qPCR assays in this study

Target Organism, Target Gene, and Citation	**Primer/Probe Sequences**	**Amplification Size (bp)**
Salmonella Typhimurium. (*invA* gene) Hein et al. (2006)	139 primer: 5'-GTGAAATTATCGCCACGTTCGGGCAA-3'; 141 primer: 5'-TCATCGCACCGTCAAAGGAACC-3' invA-1 probe: 5'-FAM-TTATTGGCGATAGCCTGGCGGTGGGTTTTGTTG-BHQ-3'	285
Shigella flexnei. (*ipaH* gene) Wang et al. (2007)	Shig-1 primer: 5'-CTTGACCGCCTTTCCGATA-3' Shig-2 primer: 5'-AGCGAAAGACTGCTGTCGAAG-3' Shigella-probe: 5'-FAM-AACAGGTCGCTGCATGGCTGGAA-BHQ-3'	117
E. coli O157:H7 (*eae* gene) Sharma (2006)	eae-forward primer: 5'-GTAAGTTACACTATAAAAGCACCGTCG-3' eae-reverse primer: 5'-TCTGTGTGGATGGTAATAAATTTTTG-3' eae-probe: 5'-FAM-AAATGGACATAGCATCAGCATAATAGGCTTGCT- BHQ-3'	106

Table 5.2 Ct values for human NoV GI obtained both with and without co-amplification of the IAC

NoV stock dilution	GI	GI+IAC	
		GI	IAC*
10^{-1}	31.3	32.23350525	33.40147781
10^{-2}	36.8	36.40215683	32.35991287
10^{-3}	40.3	Undetermined	33.26463699
10^{-4}	Undetermined	Undetermined	33.58284378
Negative control	Undetermined	Undetermined	33.0522728

Concentration of IAC: 112ng/µl.
Ct values <=35 are regarded as reliable exponential amplifications.
* 10^{-4} dilution of IAC was selected for coamplification

Table 5. 3 Ct values for human NoV GII obtained both with and without co-amplification of the IAC

NoV stock dilution	GII	GII+IAC	
		GII	IAC*
10^{-1}	25.7	21.859478	28.59191513
10^{-2}	28.2	24.42568207	26.47133446
10^{-3}	30.7	28.14121056	26.95303154
10^{-4}	35.5	31.41905403	26.90688896
10^{-5}	39.7	Undetermined	26.63337517
10^{-6}	41.4	Undetermined	26.63281822
10^{-7}	Undetermined	Undetermined	26.47579575
Negative control	Undermined	Undetermined	28.18702126

Concentration of IAC: 3368ng/µl
Ct values <=35 are regarded as reliable exponential amplifications.
*10^{-5} dilution of IAC was selected for coamplification

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