Presence of Pathogenic Bacteria and Viruses in the Daycare Environment

Abstract The number of children in daycare centers (DCCs) is rising. This increases exposure to microorganisms and infectious diseases. Little is known about which bacteria and viruses are present in the DCC environment and where they are located. In the study described in this article, the authors set out to determine the prevalence of pathogenic bacteria and viruses and to find the most contaminated fomites in DCCs. Fifteen locations in each DCC were sampled for bacteria, respiratory viruses, and gastrointestinal viruses. The locations were in the toilet, kitchen, and playroom areas and included nursery pillows, toys, and tables, among other things. Coliform bacteria were primarily found in the toilet and kitchen areas whereas nasopharyngeal bacteria were found mostly on toys and fabric surfaces in the playroom. Respiratory viruses were omnipresent in the DCC environment, especially on the toys.

Introduction
Children, especially young children aged 0–3 years, have a high frequency of infectious disease episodes (Denny, Collier, & Henderson, 1986). Daycare centers (DCCs) worldwide are ideal places for infections to spread because of the density of small children and their constant interaction. Moreover, the number of children attending DCCs is increasing. In Denmark, the vast majority of small children are cared for in center-based institutions, and in the U.S., center-based care is now the dominant form of care for young children (ChildStats.gov, 2013). Thus, it is not surprising that young children attending DCCs have more sick days than children cared for elsewhere (Bartlett et al., 1985; Fleming, Cochi, Hightower, & Broome, 1987; Uldall, 1990). This is in part due to the spread of infectious microorganisms from child to child. Other pathways of pathogen transmission may play a role, but this has not been well investigated.

Every day, the daycare environment is exposed to thousands of different microorganisms from the children, staff, and parents, but whether these fomites play a role in disease transmission is not well known. The focus in research within this field has previously been on presence of nonpathogenic bacteria or low-pathogenic bacteria in the DCC environment (Cosby et al., 2008; Laborde, Weigle, Weber, & Kotch, 1993; Staskel, Briley, Field, & Barth, 2007). Studies using culture samples have found that 10%–60% of the samples are positive for coliform bacteria depending on location. Studies using molecular methods such as quantitative polymerase chain reaction (qPCR) have determined the diversity of bacteria in DCCs and found that the main bacteria flora in the DCC environment consisted of coagulase-negative staphylococci (CoNS), Bacillus spp., and Pseudomonas-like bacteria, all of which rarely cause disease in healthy humans (Lee, Tin, & Kelley, 2007).

The majority of infections in DCCs are respiratory infections, which are mainly caused by viruses such as rhinovirus, bocavirus, adenovirus, and respiratory syncytial virus (RSV) (Fairchok et al., 2010; Martin, Fairchok, Stednick, Kuypers, & Englund, 2013; Pitkaranta et al., 2006). The presence and amount of these viruses in the DCC environment is unclear (Denny et al., 1986). A few studies have looked at influenza virus and rotavirus in the environment but these viruses are only two of the viral pathogens (Boone & Gerba, 2005; Butz, Fosarelli, Dick, Cusack, & Yolken, 1993; Keswick, Pickering, DuPont, & Woodward, 1983). Viruses causing a common cold, which is by far the most prevalent disease among young children, have not yet been the subject of thorough investigations in the DCC environment.

The aim of our study was to determine the presence and quantity of bacteria and viruses in the DCC environment and to locate the fomites with the highest prevalence of pathogens.
TABLE 1

Sampling Locations

<table>
<thead>
<tr>
<th>Room</th>
<th>Location</th>
<th>Bacteria</th>
<th>Gastrointestinal Virus</th>
<th>Respiratory Virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kitchen</td>
<td>Kitchen table</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kitchen sink</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Refrigerator door</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Playroom</td>
<td>Table upper side</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>Table underlaid</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Plastic toys</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Wooden toys</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Food toys</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>Pillows</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>Sofa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toilet</td>
<td>Toilet seat</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nursery pillow</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Toilet floor</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Water faucet</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sink</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
</tbody>
</table>

Materials and Methods

Recruitment of Institutions
Twenty-three institutions were recruited in the fall of 2011. They were randomly selected among all the public daycare centers in the municipalities of Copenhagen and Nyborg because these two municipalities had agreed to be a part of the project. Recruited institutions were all “integrated institutions” with both nursery and kindergarten divisions. The number of divisions in each institution ranged from two to seven and the number of children per institution ranged from 24 to 149. The total number of children was 1,820.

Virus Sampling and Processing
Gastrointestinal viruses were sampled from six locations and respiratory viruses were sampled from three (Table 1). A location of 10x10 cm was sampled using a 15x25 mm polyester foam swab. The swab was immersed in sterile, RNase-free water before sampling. After sampling, the swab was put into a 15-ml sterile plastic container with 5 ml Nuclisens lysis buffer. Upon arrival to the lab, the tubes were placed on a shaking table for 20 minutes and the lysis buffer was transferred to a 3.6-ml cryotube and stored at -20°C until analysis.

Nucleic Acid Extraction and qPCR
Virus DNA and RNA from the sample were extracted using a MiniMag apparatus and Nuclisens extraction reagents. The purified DNA/RNA, eluted in 100 µL of elution buffer, was stored at -80°C until qPCR amplification and analysis.

Selected samples were analyzed for the presence of 10 respiratory and 4 gastrointestinal viruses, all of which are pathogenic to healthy children. The respiratory viruses were influenza A and B, coronavirus, parainfluenzavirus, rhinovirus, RSV A and B, adenovirus, enterovirus, parechovirus, and bocavirus. The gastrointestinal viruses were norovirus genogroup G1 and G2, astrovirus, and rotavirus. qPCR was done using 10 µL of extracted nucleic acids and the following commercial multiplex PCR kits: FTD Viral Gastroenteritis and FTD Respiratory Pathogens 21 Plus using the recommended enzyme kit AgPath-ID One-Step RT-PCR Reagents. The PCR amplification and reading was done using a RotorGene Q and analysis was done using Rotorgene Software.

Bacterial Sampling and Processing
Sampling was done in February and March 2012 because a previous pilot study had shown that the winter period is the period with the highest prevalence of infectious diseases (data not shown). Fifteen predefined locations were sampled in each of the 23 DCCs (Table 1). Based on a study evaluating different sampling methods, the following sampling techniques were chosen for each location: an area of 100 cm² (10x10 cm) was sampled using 1) a sterile, cotton-tipped swab, dipped in ox broth after sampling and 2) a TV dipslide (Ibfelt, Foged, & Andersen, 2013). The TV dipslide has two sides: a nonselective side with tryptic soy agar (TSA) for total count and a violet red bile glucose agar on the other side for the isolation of Enterobacteriaceae. Moreover, the dipslide contains a neutralizer in order to neutralize traits of disinfectants and detergents. As for bacterial species presence, the results from the dipslides and the ox broth were pooled for each sample location and given as binary results depending on whether the specific bacteria were present or not.

Incubation and Identification
The dipslides were incubated for 48 hours and the ox broth for seven days at 35°C-37°C. Following incubation, the ox broth was plated onto a blood agar plate and a gram-negative selective lactose agar plate with bromothymol blue and incubated for 24 hours at 35°C-37°C. The bacteria from both the dipslide and the ox broth were identified using conventional identification and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF). MALDI-TOF was only used for potential pathogens. Total bacteria count was determined using the TSA side of the dipslide and the supplied key from the manufacturer and given as CFU/cm².

Bacteria Classification
The bacteria were divided into four groups: skin bacteria (CoNS, Micrococcus spp., Propionibacterium spp., and S. aureus), water and soil bacteria (Acinetobacter spp., Pseudomonas-like spp., Acronomonas spp., Cnamomonas spp., Bacillus spp., and mold), nasopharyngeal bacteria (S. pneumonia, Moraxella spp., and nonhemolytic streptococci), and intestinal bacteria (all Enterobacteriaceae and Enterococcus spp.). E. coli and Enterococcus spp. were used as fecal indicators. Furthermore, all potential pathogens (all fecal bacteria, S. aureus, and nasopharyngeal bacteria) were identified using MALDI-TOF and susceptibility testing was performed against cephalothin, cephalixin, and meropenem for all gram-negative rods using a disc diffusion test.
Results

Viruses
Respiratory virus presence was widespread in the daycare environment but the prevalence of the different virus species was very different. The prevalence of the 10 different respiratory viruses is depicted in Figure 1. The most prevalent virus was bocavirus (present on 81% of the sites), followed by coronavirus (77%), adenovirus (46%), and rhinovirus (29%). Other respiratory viruses such as parainfluenza, parvovirus, and influenza B were rarely found, if found at all. The presence of respiratory viruses was examined at three locations in the playroom area; the toys were the fomite with the highest general prevalence of respiratory viruses, followed by the pillows and the playroom table. Gastrointestinal viruses were less prevalent. The virus with the highest prevalence was astrovirus (12%), followed by rotavirus (2%), norovirus G1 (1.5%), and G2 (0.7%). The nursery pillow was the fomite with the highest prevalence of gastrointestinal viruses, followed by the playroom pillows and the toilet seat. The detailed information is shown in Figure 2.

Bacteria
The predominant findings were nonpathogenic bacteria, especially CoNS (333 positive locations, 97%), and various water bacteria such as *Pseudomonas*-like bacteria (159 positive spots, 46%) and *Acinetobacter* spp. (61 positive spots, 18%). We did not find any *S. aureus*. As for fecal indicator bacteria, only 2 out of 345 (0.2%) locations tested positive for *E. coli* and one location (0.1%) tested positive for Enterococci. These locations were a toilet seat, a kitchen sink, and a nursery pillow, but not all in the same institution. When counting all coliform bacteria, 40 locations (11.6%) were found positive. Of these, 15 locations were in kitchen areas, 15 locations were in toilet areas, and 10 locations were in playroom areas. A more detailed outline of the coliform-positive locations is shown in Figure 3.

Nasopharyngeal bacteria were present on 58 locations (16.8%). Species were dominated by nonhemolytic streptococci (40 locations, 12%) and *Aerococcus* sp. (15 locations, 4%) while one location was found positive for *S. pneumoniae* and two locations for *Moraxella* sp. In contrast to the coliform bac-
teria, most positive locations were found in the playroom area, especially on the toys. A more detailed outline of the positive fomites is shown in Figure 4.

We found no multiresistant (extended-spectrum beta-lactamase or Carbapenem-resistant) E. coli or K. pneumoniae in the DCC environment.

Discussion

The bacteria found in the DCC environment were mainly nonpathogenic and we only found a few fecal bacteria and nasopharyngeal pathogen bacteria. A study done in 2007 by Lee and co-authors investigated the bacterial diversity in a DCC through a combination of cultures and 16S rRNA sequencing. They found that the most prevalent bacteria from culture plates were Bacillus spp., Staphylococcus sp., and Pseudomonas sp. while 16S sequencing analysis was dominated by Pseudomonas sp. and Oxalobacteria sp. In our study, the goal was to locate viable bacteria using culture methods and our results are quite similar to those by Lee and co-authors. We observed a very low prevalence of fecal indicators compared to many other studies, e.g., by Laborde and co-authors, who found frequencies of 20%-50% positive samples for fecal coliforms on toys, sinks, and tables in toddler classrooms (Laborde, Weigle, Weber, Sobsey, & Kotch, 1994). Ekanem and co-authors (1983) found rates of isolation of fecal coliforms of 13% from classroom objects. If looking solely at the fecal indicators E. coli and Enterococcus spp., the prevalence in our study was very low. But if we include all coliforms, the prevalence was 11.6%, similar to that found by Ekanem and co-authors. The sampling locations and methods were not similar, however, which complicates the comparison.

We found more respiratory viruses than bacteria in the environment. The respiratory virus diversity and the prevalence rates on the surfaces correspond well to the rates found in child airways in other studies (Bonfin et al., 2011; Fairchok et al., 2010; Martin et al., 2013). These studies found rhinovirus, RSV, coronavirus, and adenovirus to be the most prevalent type of virus in children with respiratory tract infections. This corresponds well with our results, although bocavirus was the most prevalent virus, followed by coronavirus, adenovirus, and rhinovirus. RSV detection was low, probably because the children suffering from RSV pneumonia are often severely ill and are therefore not sent to daycare. Bocavirus is a rather newly discovered virus and its causal role in respiratory tract infections is still unclear (Allander et al., 2005; Schildgen et al., 2008). The high prevalence of bocavirus is in accordance with other studies but the number may be high due to
asymptomatic shedding of the virus and not actual infections (Schildgen et al., 2008).

Gastrointestinal virus presence in the environment ranged from a positivity rate of approximately 15% for astrovirus to only a few positive samples for rota- and norovirus. As for rota- and norovirus, their presence is highly outbreak-related and samples were taken when no outbreaks occurred in the DCCs. It is therefore not surprising to find low viral numbers and this is in accordance with other studies finding low rates of rota-virus in the daycare environment and significantly higher rates during outbreaks (Boxman et al., 2011; Butz et al., 1993; Wilde, Van, Pickering, Eiden, & Yolken, 1992). Had our samples been taken during an outbreak period, the rates may have been higher.

Viruses can be difficult to show in environmental samples because the amount of virus is low. In this study we used a sampling method that was developed and tested by our group prior to sampling (data not yet published), combined with a commercial, real-time PCR kit that covered the most prevalent respiratory and gastrointestinal viruses in children. The sensitivity had been tested in our prior study using norovirus as a model virus and we found the detection limit to be approximately 100 virus copies/cm². This may differ with other types of virus than norovirus but generally the method is sensitive enough to show a virus amount in the environment that is pathogenic. In contrast, the method may overestimate the virus amount because it also detects inactivated viruses and virus RNA/DNA fragments, which are not pathogenic. Thus, we may have either overestimated or underestimated the amount of pathogenic virus in the environment, but the method we used is, in our opinion, the best method available today.

Conclusion
In conclusion, our study showed that bacteria and viruses, especially respiratory viruses, are omnipresent in the daycare environment. Toys were particularly contaminated with respiratory viruses whereas fecal bacteria and viruses were mainly found in the toilet and kitchen areas. Toys may be one of the most contaminated fomites and may contribute to indirect pathogen transmission. Our group is currently conducting a study to examine the effect of toy washing on microorganism presence and infectious diseases in DCCs, the results of which will be published when that study is finished.

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