

HYPOCHLORITE KILLING OF COMMUNITY-ASSOCIATED METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

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Abstract: We tested in vitro hypochlorite (bleach) killing of community-associated methicillin-resistant *Staphylococcus aureus* isolates to determine optimal concentration and duration. For all isolates maximal killing, >3-log decrease in colony forming units (CFU), was found after 5 minutes in 2.5 $\mu\text{L}/\text{mL}$ bleach. We estimate that 2.5 $\mu\text{L}/\text{mL}$ bleach is approximately one-half cup of bleach in one-quarter tub of water.

Key Words: *Staphylococcus aureus*, MRSA, hypochlorite, bleach bath

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Over the past several years, clinics and hospitals have seen a meteoric rise in the number of infections caused by community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA).^{1,2} Both systemic and superficial infections have been reported.^{3,4} Recurrent CA-MRSA skin infections have become a common clinical problem.⁵

Treatment of individual outbreaks of furuncles is usually accomplished by drainage with or without oral antibiotic therapy.³ However, furuncles tend to recur, and prevention of these recurrences has proven to be a vexing problem. Patients with recurrent furunculosis are frequently carriers of CA-MRSA; carriage can be in the anterior nares, the perianal area, and/or the skin in general.^{6,7}

Prevention of recurrent outbreaks has been attempted by various means. Many physicians prescribe a course of intranasal mupirocin ointment to eradicate nasal carriage of CA-MRSA.⁸ In an attempt to decrease the colony count of *S. aureus* on the skin, physicians have resorted to having patients wash with PhisoHex or Hibiclens,⁹ or to recommending "bleach baths." There are no studies of the concentration of bleach solution needed to kill CA-MRSA or the duration of exposure required. Bathing in bleach at a concentration of 1 teaspoon bleach per gallon of water (1.3 $\mu\text{L}/\text{mL}$ vol/vol) for 15 minutes twice a week has been noted anecdotally to correlate with fewer recurrences of skin infection.¹⁰ Patients referred to our clinic have been recommended a variety of regimens by other providers, from as weak as "one capful" of bleach in a full tub of water to as strong as 1 full cup of bleach in a quarter tub of water, and have been asked to sit in these solutions for as long as 20-30 minutes at a time. This study was designed to determine the optimal concentration and exposure time of hypochlorite solutions on killing CA-MRSA in vitro.

MATERIALS AND METHODS

Bacterial Strains and Growth. *S. aureus* laboratory strain, Reynolds, (provided by Dr. Jean Lee, Channing Laboratory, Harvard Medical School, Boston, MA) was used as a control. CA-MRSA clinical isolates were recovered as part of routine patient care from patients at the Children's Hospital of The King's Daughters, Norfolk, VA, in

accordance with Eastern Virginia Medical School IRB protocol #06-04-WC-0040. Five isolates were from children with invasive MRSA infections and 5 isolates were from children with MRSA colonization. Invasive isolates were recovered from blood, bone, or joint cultures. Colonizing isolates were recovered by nasopharyngeal swab as part of routine colonization screening. Bacteria were grown on 2% NaCl Columbia agar plates (Becton Dickinson, Franklin Lakes, NJ) overnight at 37°C. The investigators felt that growth on agar would more closely simulate growth on a solid surface (ie, skin) than growth in liquid media. Bacterial colonies were scraped from agar plates and suspended in sterile phosphate-buffered saline (PBS). The concentration of bacteria was standardized by optical densitometry to 10⁹ colony forming units (CFU)/mL. Light microscopy confirmed that a uniform suspension of bacteria was achieved with minimal clumping.

Hypochlorite Killing Assays. One milliliter of bacterial suspensions (10⁹ CFU) were sedimented by centrifugation and resuspended in municipal tap water or municipal tap water with hypochlorite. Clorox bleach (The Clorox Co., Oakland, CA), a 6% hypochlorite solution, was diluted in municipal tap water to 2.5 $\mu\text{L}/\text{mL}$ (vol/vol), unless otherwise described. Bacterial suspensions were then incu-

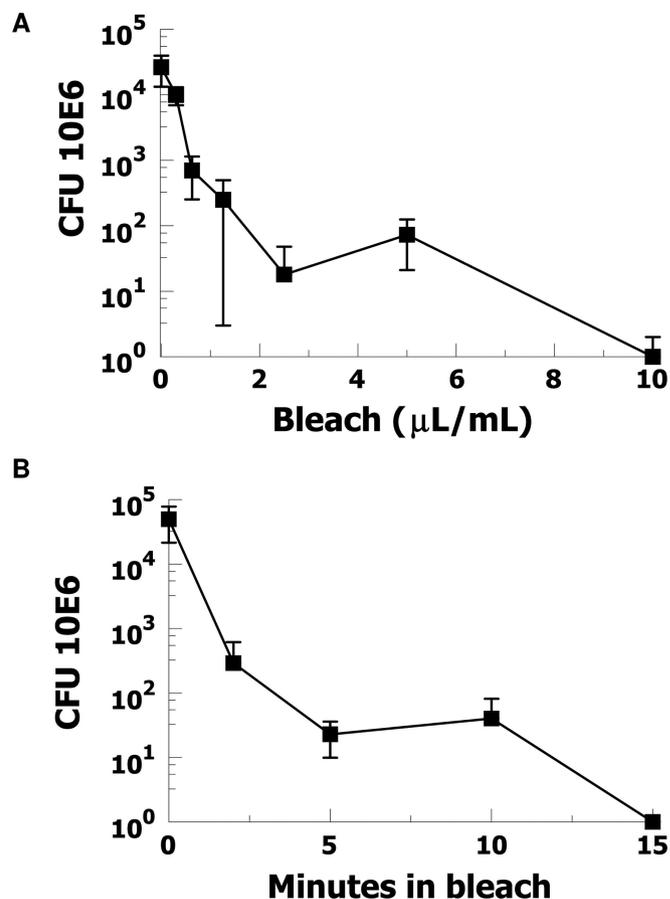


FIGURE 1. Dose-response killing of control *S. aureus* strain, Reynolds, with increasing concentrations of hypochlorite incubated for 10 minutes at 37°C (A). Mean colony counts of remaining live bacteria on logarithmic scale ($n = 4$, $\pm\text{SD}$). Time course for the response of control *S. aureus* strain, Reynolds, for increasing incubation times, 2.5 $\mu\text{L}/\text{mL}$ hypochlorite solutions incubated at 37°C (B). Average colony counts of remaining live bacteria on logarithmic scale ($n = 5$, $\pm\text{SD}$).

bated in a 37°C waterbath with agitation for 10 minutes, unless otherwise described. Bacteria were then sedimented by centrifugation and serially washed 3 times with 5 mL PBS. Resuspended bacteria were then serially diluted with PBS, and 0.1 mL of each dilution was plated onto agar. Agar plates were incubated overnight at 37°C and colony counts were performed for plates demonstrating 30–300 colonies the following day and corrected for the dilution factor.

Statistical Analysis. The values of replicate experiments were averaged, from which means and standard deviations were calculated. Statistical differences were evaluated by Student *t* test.

RESULTS

Hypochlorite Dose-Dependent Killing. Hypochlorite (10 minutes, 37°C) demonstrated dose-dependent killing of *S. aureus* (control strain) compared with municipal tap water without hypochlorite. A greater than 3-log decrease in surviving *S. aureus* was achieved with a 2.5- μ L/mL dilution of bleach and a greater than 4-log decrease was achieved with a 10- μ L/mL dilution of bleach. By contrast, a decrease of 2 logs was achieved with a 1.2- μ L/mL dilution of bleach (Fig. 1A). The 2.5- μ L/mL dilution of bleach was chosen for further study because >3 logs of *S. aureus* killing was achieved and our clinical experience suggested that this concentration was not excessively noxious for children.

Hypochlorite Time-Dependent Killing. Hypochlorite (2.5 μ L/mL, 37°C) demonstrated time-dependent killing of *S. aureus* (control strain) compared with municipal tap water without hypochlorite. A greater than 3-log decrease in surviving *S. aureus* was achieved after a 5-minute incubation and a >4-log decrease was achieved after a 15-minute incubation (Fig. 1B).

Hypochlorite Killing of Clinical CA-MRSA. All 10 CA-MRSA isolates showed >3 logs of killing after hypochlorite (2.5 μ L/mL, 10 minutes, 37°C) compared with tap water alone. The range of killing for the different CA-MRSA isolates was 99.94–100% and not statistically different (*n* = 3). No difference was found for susceptibility to hypochlorite comparing 5 colonizing isolates with 5 invasive isolates.

DISCUSSION

CA-MRSA has become a relatively common problem in recent years.⁷ Control measures for recurrent CA-MRSA skin infections, including recurrent furunculosis, are needed. In the absence of clinical trial data, clinicians have been using innovative measures, such as bleach baths. The present study provides evidence that weak hypochlorite solutions are effective in killing staphylococcal isolates in vitro, including invasive and colonizing clinical isolates of MRSA.

We found that a 2.5- μ L/mL dilution of bleach was rapidly effective in killing invasive and colonizing clinical CA-MRSA isolates, decreasing viable colonies by >3 logs (>99.9%) within 5 minutes. A 2.5- μ L/mL dilution of bleach is approximately equivalent to one-half cup (120 mL) of bleach in one-quarter filled tub of water (13 gallons of water in a 50-gallon standard tub). Whether this would translate into decreased frequency of staphylococcal skin infections in patients with recurrent furunculosis remains to be seen, and requires a clinical trial.

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COMMENTARY: PREVENTION OF RECURRENT STAPHYLOCOCCAL INFECTIONS

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Key Words: *Staphylococcus aureus*, recurrent infection, sodium hypochlorite

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Methicillin-resistant *Staphylococcus aureus* (MRSA) is an established pathogen in many communities of the United States.¹ Community-acquired MRSA (CA-MRSA) infections have changed several aspects of staphylococcal infections in children including the epidemiology, clinical manifestations, laboratory diagnosis, treatment, and prevention.² The incidence of *S. aureus* infections in children is not clearly known, but in a Centers for Disease Control and Prevention (CDC) study the incidence of CA-MRSA infections in 2001–2002 for white children and black children <2 years old was approximately 16 and 70 of 100,000 in Atlanta and 18 and 40 of 100,000 in Baltimore, respectively.³ In 2005 the annual incidence of community-onset MRSA infections among children <5 years of age in San Francisco was 227 of 100,000.⁴

At Texas Children's Hospital (TCH) in Houston, CA-MRSA isolates account for more than 75% of CA-*S. aureus* isolates recovered from children.⁵ Furthermore, the absolute number of CA-*S. aureus* infections increased over the years, doubling in number from 2001 to 2004. Skin and soft tissue infections (SSI), such as abscesses and cellulitis account for over 95% of the type of infections caused by CA-MRSA in children. Any area of the body may be infected but the groin, buttock, and extremities are the most common sites. Osteomyelitis, septic arthritis, pneumonia with empyema, necrotizing pneumonia, and pyomyositis/myositis lead the list of the invasive infections.⁵⁻⁹ Thus, *S. aureus* infection is a major public health problem for normal healthy children in the United States and has become a major cost factor for health-care dollars.¹⁰

About 30% of healthy people are colonized with *S. aureus* in the anterior nares but nasal carriage of MRSA has been low in community surveillance studies in the past.¹¹ However, in 2 recent studies MRSA nasal carriage was 9% and 22% in children at well visits in Nashville, TN¹¹ and on admission to a children's hospital in Corpus Christi, TX,¹² respectively. What accounts for this increase in MRSA nasal colonization is clearly a pressing area of investigation. *S. aureus* isolates can be classified into 11 main pulsotypes (USA100–USA1100) on the basis of pulse field gel electrophoresis banding of DNA after digestion.¹³ Using this classification system the USA300 CA-MRSA clone, which typically carries the staphylococcal chromosome cassette type IV (SCC*mec* IV) and the genes encoding for the exotoxin Pantone-Valentine leukocidin (pvl), is the dominant pulsotype among CA-MRSA isolates in the United States.¹⁴ Why the USA300 clone is particularly capable of spreading rapidly in the community is not known. It is conceivable that USA300 has some enhanced ability to colonize the nose or other body sites, such as the groin area, and to spread from person to person effectively.¹⁵

Recurrent skin infections with CA-MRSA are common.¹ In a study among adults, a recurrence rate of 15% was noted¹⁶; 5% of children had recurrent infections in a report from Minnesota.¹⁷ In a prospective study, preliminary data suggest that 19% of children had recurrent skin infections.¹⁸ At TCH among patients with culture confirmed *S. aureus* infections from August 1, 2001 to November 2006, 474 of 9420 (5%) children had 2 or more confirmed *S. aureus* infections.¹⁹ Furthermore, multiple family members, such as a sibling or parent are often infected, although there are no data on how frequently this occurs.²⁰

The optimal strategy to prevent recurrent CA-*S. aureus* infections has not been established.¹¹ Although a panel of experts has provided key recommendations for the prevention of skin and soft tissue infections, these measures have not been tested for their effectiveness.²¹ In addition to adhering to routine hygienic measures, applying mupirocin, a topical antibiotic active against most MRSA isolates, to the anterior nares several times a day for a week may reduce nasal colonization, and possibly recurrent infections, and may be recommended in selected circumstances.²¹ At TCH a strategy for preventing recurrent CA-*S. aureus* skin and soft tissue infections has included the use of sodium hypochlorite (Clorox)

in baths in addition to the traditional measures outlined by the expert panel. Parents are instructed to have their children take a bath twice a week for 15 minutes in water to which a teaspoon of regular strength Clorox has been added for each gallon of bath water. This approach has been recommended for many years by dermatologists for eczema patients with recurrent *S. aureus* skin infections and also has been used in hydrotherapy water in burn units.²²⁻²⁴ We have found this measure to be safe and anecdotally helpful in reducing the frequency of recurrent CA-MRSA skin infections. However, there are no data documenting the efficacy and safety of sodium hypochlorite baths in the prevention of *S. aureus* skin and soft tissue infections.

Fisher and colleagues²⁵ report in this issue of the journal that in a series of in vitro experiments, sodium hypochlorite (bleach) at a concentration of 2.5 $\mu\text{L}/\text{mL}$ of tap water resulted in maximal killing of several clinical strains of CA-MRSA recovered from children at their hospital after 5 minutes of exposure. This dilution is about equivalent to adding one-half cup of household bleach in a quarter tub of water. The study by Fisher et al²⁵ does provide some basis for using the dilution of sodium hypochlorite being recommended. However, only a randomized trial will determine if adding "bleach baths" to routine measures is safe and effective in reducing recurrent *S. aureus* infections in children. In fact, a randomized study including sodium hypochlorite is being conducted to assess this measure in atopic dermatitis www.clintrials.gov (NCT00179959). Hopefully, this and other controlled trials will lead to optimal strategies for prevention of recurrent *S. aureus* infections, but with the dream that at some point an efficacious vaccine to prevent *S. aureus* infections will be developed.²⁶

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PROTRACTED FEVER WITH CELLULITIS-LIKE REACTION IN PNEUMOCOCCAL POLYSACCHARIDE-VACCINATED CHILDREN

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Abstract: Side effects of the 23-valent pneumococcal polysaccharide vaccine (PPV23) are regarded as mild and uncommon in adults.

In our clinical experience, however, protracted fever with a cellulitis-like reaction after PPV23 vaccination is not rare in pediatric patients. We reviewed the records of 17 children with those clinical findings after PPV23 vaccination.

Key Words: Pneumovax, 23-valent pneumococcal polysaccharide vaccine, local reaction, fever, pediatric

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Although 7-valent pneumococcal conjugate vaccine (PCV7) has been marketed since 2000 for use in children, the 23-valent pneumococcal polysaccharide vaccine (PPV23) is recommended for older children who are thought to be at high risk of pneumococcal disease.¹ The PPV23 contains antigens of 23 serotypes of pneumococci that cause approximately 90% of bacteremic pneumococcal diseases. It covers 87% of the penicillin-resistant serotypes of *Streptococcus pneumoniae* in Taiwan.² Because PCV7 has not yet been included into the routine vaccination schedule in Taiwan, pediatricians commonly use PPV23 in children aged >2 years.

Approximately half of the individuals who receive pneumococcal vaccine, regardless of age, develop mild, local side effects (eg, pain at the injection site, erythema, and swelling). These reactions usually persist for <48 hours.^{3,4} In postmarketing reports, cellulitis-like reactions at the PPV23 injection site have rarely been reported; between 1989 and 2002, when approximately 43 million doses were distributed, the annual reported incidence was <2 of 100,000 doses (data from Merck Company).

In our clinical experience, systemic febrile reaction after PPV23 vaccination is not so rare in pediatric patients. We report 17 children hospitalized with protracted fever and a cellulitis-like reaction after PPV23 vaccination, describing the clinical course of this adverse reaction.

SUBJECTS AND METHODS

We searched the database of the Vaccine Injury Compensation Program (VICP) of Taiwan's Centers for Disease Control to identify all patients with severe adverse reaction after PPV23 vaccination (Pneumovax 23, Merck and Company, Whitehouse Station, NJ) who applied for benefits from January 2001 to February 2007. Our search yielded records for a total of 17 patients approved for compensation. All were younger than 12 years of age. There were no adults who applied for compensation after PPV23 inoculation during that period.

All children in the study were previously healthy. They had been given an intramuscular injection of 0.5 mL of PPV23. All were reported to have a fever of >38.5°C with a severe local reaction at the injection site, and all were hospitalized for treatment. Data extracted from the charts included gender, age, inoculation site,

clinical presentation (including signs and symptoms, time of onset after injection, local findings, body temperature, duration of fever, and duration of other symptoms), duration of hospitalization, white blood cell count, absolute neutrophil count, C-reactive protein value, and blood culture results. The laboratory data reported reflect the values obtained on admission. We also documented imaging studies, any invasive procedures, and antibiotic therapy that was given.

RESULTS

The mean age of the 17 children was 62.8 months (range, 24–138 months) (Table 1, <http://links.lww.com/A480>). Nine were boys and 8 were girls. Nine children were given the vaccine in the deltoid region, 4 into the anterolateral thigh, and 4 into the gluteal region. Swelling occurred at a mean of 8.9 hours (range, 3–24 hours) and fever at 14.7 hours (range, 4–32 hours) after injection. Erythema and local heat persisted for up to 17 days, tenderness for 14 days, and induration for 21 days. The pain was so severe in some children as to restrict the range of motion in the injected limb (arm for deltoid injections, leg for gluteal or thigh injections). The mean maximum length of the lesion was 10.4 cm (range, 5–20 cm). The mean maximum body temperature was 39.9°C (range, 39–41.7°C), with a mean of 4.9 days until defervescence (range, 3–7 days). Other symptoms included abdominal pain and vomiting in 5, headache that resulted in a lumbar puncture in 1, febrile convulsion prompting brain computed tomography in 1, an eschar at the injection site, an “abscess-like” formation in 3, systemic inflammatory response syndrome in 2, and what appeared to be necrotizing fasciitis in 1.

The mean white blood cell count was $20,414 \times 10^9/L$ (range, 10, 500–57,600), with 76% neutrophils (range, 58–93%), and the mean C-reactive protein concentration was 150 mg/L (range, 22.9–275.3 mg/L). All 17 children had blood cultures drawn, which were uniformly negative. Two of the 3 children whose doctors reported an abscess-like formation underwent needle aspiration of the lesions. Only a minimal amount of fluid was drained; there was no pus. This material was not Gram-stained, but cultures were negative. Joints adjacent to the reactions were evaluated by radiographs in 6 children, but no abnormalities were seen. The mean length of hospital stay was 7.1 days (range, 3–17 days). During hospitalization, all children were treated with intravenous antibiotics, 6 with 1 drug, 8 with 2 drugs, and 3 with 3 antibiotics. Vancomycin was used in 4 children and teicoplanin in 1. The mean duration of antibiotic treatment (intravenous and oral) was 10.6 days (range, 3–20 days).

DISCUSSION

The VICP of Taiwan’s Centers for Disease Control was established in 1992. Taiwanese people can file a claim if they believe that they have been injured by a vaccine resulting in a hospital stay, surgery, or death. During the period of 1995–2007, the VICP received 196 applications for benefits. Among the 109 patients approved for compensation, 17 patients (15.6%) were as a result from receiving PPV23, which was the second most injurious vaccine.

Our study has documented protracted fever and a cellulitis-like reaction after PPV23 vaccination in children, which we believe is attributable solely to the vaccine. It was particularly noteworthy that the clinical course in these 17 cases was different from that of bacterial cellulitis. The swelling and the erythema began on average 9 hours after PPV23 injection, which is far shorter than would be expected if the injection had caused cellulitis. Similarly, the onset of fever after vaccination was much sooner than it would have been for bacterial cellulitis at the injection site. Severe cellulitis may lead to a localized abscess and is sometimes associated with bacteremia,⁵ none of which was seen in our series. The reports of abscess-like lesions were not in fact consistent with a true abscess. Even though

antibiotics were given, the PPV23-induced reactions did not seem to respond to treatment. They abated gradually.

Fewer than 10 cases have been reported documenting severe systemic or dermatologic reactions in adults receiving their first PPV inoculation.^{6–8} In the pediatric population, adverse reactions to PPV have mostly been attributed to the 14-valent vaccine. Local soreness and/or induration at the injection site was reported in 29–44% of 826 vaccinated children in 2 large clinical trials, and fever greater than 37.7°C (99.9°F) was reported in 3–19% of vaccinated children. Fever was usually confined to the 24-hour period after vaccination. Fever greater than 38.9°C (102°F) and marked local swelling have been reported, but they are unusual.⁹ Our finding of 17 children with high fever and marked local reaction after PPV23 immunization suggests that the reaction is not rare. The fever can persist longer than 24 hours. We cannot calculate the precise incidence of this side effect in children, as the total number of children given PPV23 vaccine in Taiwan during the study period cannot be obtained. In addition, the incidence might be underestimated because we evaluated only those children whose parents applied for compensation from VICP. Based on our results, however, we believe further study of the PPV23 vaccine in pediatric patients should be conducted to determine the risk of a severe reaction.

There was no correlation between the site of injection, gender, or age and the severity of the reaction. The depth of an intramuscular injection has been associated with differences in local or systemic reactions after vaccination.^{3,10} The length and bore size of the needles were not recorded for the patients in our series, so we could not evaluate this factor.

In conclusion, our data lead us to believe that PPV23 can induce protracted fever and a cellulitis-like reaction in some pediatric patients. Healthcare professionals should be aware of the possibility of such adverse reactions. Certainly if there is bacterial contamination of the needle, syringe, or vaccine, cellulitis is a possibility, but such an infection would not show up as early as the reaction we have described. It is likely that neither hospitalization nor antibiotics are necessary for the early severe reaction we have described, although our study was not designed to answer this question. As always, clinical judgment is the key to both diagnosis and management.

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THE ROLE OF RESPIRATORY VIRAL INFECTIONS AMONG CHILDREN HOSPITALIZED FOR COMMUNITY-ACQUIRED PNEUMONIA IN A DEVELOPING COUNTRY

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Abstract: We report an investigation for 16 bacteria and viruses among 184 children hospitalized with pneumonia in Salvador, Brazil. Etiology was established in 144 (78%) cases. Viral, bacterial, and mixed infections were found in 110 (60%), 77 (42%), and 52 (28%) patients, respectively. Rhinovirus (21%) and *Streptococcus pneumoniae* (21%) were the most common pathogens. Our results demonstrate the importance of viral and pneumococcal infections among those patients.

Key Words: etiology, respiratory syncytial virus, rhinovirus, *Streptococcus pneumoniae*

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In most developing countries, community-acquired pneumonia (CAP) is the leading cause of hospitalization and death among children under 5 years of age, despite its documented reduction of mortality because of the development of antimicrobial therapy, CAP management guidelines, and effective vaccines.¹ The majority of CAP cases readily respond to appropriate antibiotic therapy and are considered bacterial rather than viral infections.² Despite its worldwide impact, data on childhood CAP causative organisms are few,³ mainly owing to the difficulty of establishing the etiology of CAP reliably.

We present the results of a thorough investigation involving children with CAP by means of several techniques.

METHODS

From September 2003 to May 2005, 322 consecutive hospitalized children with CAP were evaluated at Professor Hosannah de Oliveira Pediatric Center, Federal University of Bahia, Salvador, Northeast Brazil. The diagnosis was based on respiratory complaints and fever, or difficulty in breathing and pulmonary infiltrates compatible with pneumonia on the chest radiograph at admission. A second chest radiograph (2–4 weeks after recruitment) was taken to confirm resolution of pneumonia. A pediatric radiologist, blind to clinical information, read the recruitment and follow-up chest radiographs. The inclusion criteria included age <5 years and radiologically diagnosed pneumonia. Initially, 322 patients were evaluated. Exclusion was due to refusal to give informed consent (28), child born to a HIV-infected woman (6), chronic lung disease except asthma (6), varicella (3), and immunodeficiency (2). Pneumonia was confirmed by the pediatric radiologist in 206 patients, of whom 184 had radiologic resolution documented at follow-up. Four patients had 2 episodes of CAP, which were analyzed separately in the study, because the interval between the episodes was longer than 2 months and resolution was radiologically documented. Therefore, the study group comprised 184 CAP episodes.

On admission, a blood sample was collected for serologic analysis (n = 184) and blood culture (n = 173). A nasopharyngeal sample was aspirated through a nostril and kept under –70°C until virologic tests (n = 181) were done. Every recruited child was re-evaluated 2–4 weeks after admission when the second blood sample (n = 181) and the follow-up chest radiograph (n = 184) were taken and a clinical examination was carried out.

Viral antigens in nasopharyngeal aspirates and virus-specific paired serum antibody titers [influenza A and B viruses, respiratory syncytial virus (RSV), parainfluenza virus type 1, 2, and 3, and adenovirus] were searched for, and 2 reverse transcription-polymerase chain reaction (PCR) assays were used for detection of rhinoviruses as described earlier.³ The virologic studies were carried out at the Department of Virology, Turku University Hospital, Turku, Finland.

In acute and convalescent serum samples antibodies against *Streptococcus pneumoniae*, nontypable *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Chlamydia pneumoniae* were measured and in admission samples *Chlamydia trachomatis* IgG antibodies were determined as previously reported.³ PCR was used for the detection of pneumococcal DNA in blood buffy-coat (n = 178)⁴ after extraction of DNA using QIAamp DNA Blood Mini-Kit (Qiagen, Hilden, Germany). These tests were carried out at the National Public Health Institute, Oulu, Finland. IgM antibodies to *Mycoplasma pneumoniae* were measured using a commercial EIA kit (Platelia; Bio-Rad, Marnes la Coquette, France) at the Centro de Pesquisa Gonçalo Muniz, Fiocruz, Salvador, Brazil.³

The frequency distribution of viral, bacterial, and of any potential pathogen was analyzed according to age. The corrected χ^2 or Fisher exact test, as appropriate, was used to compare the proportions among the age groups. As a continuous variable, age was evaluated using the Student *t* test (distribution was normal), and the 95% confidence interval of the mean difference (95% CI MD) was calculated. All tests were 2-tailed with a significance level of 0.05. SPSS software (version 9.0) was used for analysis. The study was approved by the Ethics Committee of the Federal University of Bahia.

RESULTS

The study included 109 (59%) boys and 75 (41%) girls. Their median age was 1.6 years (range, 26 days–4.9 years). Evidence of an etiologic agent was obtained in 144 (78%) of 184 cases. Viral infection was found in 110 (60%) and bacterial infection in 77 (42%) pneumonia cases. A mixed viral-bacterial infection was seen

TABLE 1. Etiology of Community-Acquired Pneumonia According to Age Strata Among Brazilian Hospitalized Children (n = 184)

Age (mo)	Total No.	Etiology Detected			Total Detected
		Viral	Bacterial	Viral-Bacterial	
<2	6	2 (33)	5 (83)	1 (17)	6 (100)
2–11	52	35 (67)	17 (33)	11 (21)	41 (79)
12–23	53	35 (66)	22 (41)	15 (28)	42 (79)
24–59	73	38 (52)	33 (45)	16 (22)	55 (75)
Total	184	110 (60)	77 (42)	43 (23)	144 (78)

Total detected refers to cases in which etiology was determined. The numbers in parentheses are percentages.

in 43 (23%) patients. A sole viral infection was identified in 67 (36%) and a sole bacterial infection in 34 (18%) cases. Table 1 shows the frequency of the detected etiologic agent according to agent group and age strata. The difference of viral or bacterial infection frequency was not significant when children aged <2 years were compared with children aged >2 years (data not shown). No patients had received any pneumococcal vaccine, and 149 (93.7%) of 159 who presented the vaccination card had received *H. influenzae* type b vaccine.

Rhinovirus (21%), parainfluenza viruses (17%), RSV (15%), influenza A and B viruses (9%), enterovirus (5%), and adenovirus (3%) were found as causative viruses. Children infected with RSV were younger than those without RSV infection (16.7 ± 11 versus 23 ± 15.5 months; $P = 0.01$; 95% CI MD: 1.4–11.2 months). None of the patients aged >2 years had adenovirus infection. The difference in age between children with adenovirus infection and those without it was significant (12.5 ± 4.5 versus 22.3 ± 15.2 months; $P = 0.001$; 95% CI MD: 5.0–14.6 months). No age difference was found in the distribution of other viral infections.

S. pneumoniae (21%), *H. influenzae* (8%), *M. pneumoniae* (8%), *C. trachomatis* (4%), *M. catarrhalis* (3%), and *C. pneumoniae* (1%) were found as causative bacteria. Blood culture yielded 9 (5%) pathogenic bacteria in 173 cases, and they all were *S. pneumoniae*. Of the 39 pneumococcal pneumonia cases, pneumococcal serologic tests were positive in 29 (74%) and PCR was positive in 5 (13%). *M. pneumoniae* infections were significantly more common in children ≥ 2 years of age (15%) than in those <2 years of age (3%) ($P = 0.005$). The mean ages of patients infected with and without *M. pneumoniae* were 35.3 (SD 15.6) and 20.9 (SD 14.5) months, respectively ($P = 0.001$; 95% CI MD: 6.3–22.3 months). *C. trachomatis* infections were significantly more frequent among children under 1 year of age (12% versus 1%; $P = 0.001$). The mean age of the 8 children with *C. trachomatis* infection was 7.7 months (SD 16.6), whereas the mean age of children in the other 176 cases was 22.6 months (SD 14.7) ($P = 0.006$; 95% CI MD: 4.4–25.4 months). *M. catarrhalis* infection was significantly more common among older children (36.0 ± 17.3 versus 21.6 ± 14.9 months; $P = 0.04$; 95% CI MD: 1–27.7 months). The child with a positive test for *C. pneumoniae* was 50 months old. No significant differences were seen in the occurrence of pneumococcal and *H. influenzae* infection among the different age groups.

DISCUSSION

Our results indicate that viral infection is more frequent than bacterial infection in all age strata from 2 months of age onward among children hospitalized with CAP in a developing country.⁵ *S. pneumoniae* was the most frequent bacterial pathogen in all age groups. The decrease in the incidence of radiologically confirmed

pneumonia was 17.7% in intention-to-treat analysis of a clinical trial of conjugate pneumococcal vaccines.⁶ On the basis of our results, we estimate that 21% of the patients had pneumococcal infection. Therefore, it is highly probable that *S. pneumoniae* infects one-fifth of children with CAP.

Rhinovirus, notably, was the most frequent viral agent detected. Rhinovirus infections are increasingly seen among children with CAP, partly explainable by the use of new sensitive PCR techniques.⁷ Rhinovirus had been considered a benign and sole upper airway pathogen for a long time, but it has been shown to infect lower respiratory tract cells and induce cytotoxicity and a local inflammatory response.⁸ Rhinovirus may be present in asymptomatic individuals.⁷ As we searched for rhinovirus in the nasopharyngeal secretions and not in lung tissue, we cannot rule out that several of the rhinovirus-positive patients were carriers.

Parainfluenza viruses were the second most common detected virus. The high frequency of human parainfluenza virus-associated hospitalizations among children <5 years of age has been reported from the United States.⁹ Influenza viruses were identified in 9% of our cases. Although pneumonia is detected in a minority of children with influenza infection, among children with CAP influenza virus was found in 16% of all cases and such rate increased by 100% when viral PCR was used to detect influenza.¹⁰ Parainfluenza and influenza viruses played an important role in our cases, but more cases could have been detected if we had used PCR to search for them, as well as for adenovirus.

Infection due to *M. pneumoniae* is usually considered among children aged >5 years, but a high frequency of CAP due to *M. pneumoniae* has been reported in children aged >18 months.¹¹ We detected infection due to *M. pneumoniae* in children <2 years of age; nonetheless, it was much more common among patients aged >2 years. Interestingly, we detected infection due to *C. trachomatis* in children >1 year of age, and the mean age of the infected children was 7.7 ± 16.6 months. Our data suggest that this infection is not restricted to the first few months of life.

Newly respiratory viruses identified as causative agents of pneumonia in children were not searched for in this study: human metapneumovirus, 2 human coronaviruses, and human bocavirus. Nonetheless, to the best of our knowledge, this is the first study conducted in a developing country using several sensitive diagnostic tests to search for pathogens causing viral and bacterial infections in children hospitalized for CAP. We found evidence of a high frequency of respiratory viral infections. Further studies are needed of respiratory viral infections in developing countries.

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VITAMIN D DEFICIENCY AND INSUFFICIENCY IN CHILDREN WITH TUBERCULOSIS

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Abstract: We examined the prevalence of vitamin D deficiency and insufficiency in children attending our tuberculosis (TB) clinic during a 2-year period. Sixty-four patients were included with active TB ($n = 26$) or latent TB infection ($n = 38$). Eighty-six percent ($n = 55$) were either vitamin D deficient (serum 25-hydroxyvitamin D <20 nmol/L) or insufficient (serum 25-hydroxyvitamin D <75 nmol/L). Only 1 child with active TB was vitamin D replete.

Key Words: tuberculosis, vitamin D, deficiency, insufficiency, children

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Tuberculosis (TB) rates in London are rising yearly, particularly among immigrants from sub-Saharan Africa and the Indian subcontinent.¹ The risk factors for TB are multifactorial but one contributory factor may be vitamin D deficiency, notably among groups in whom diet, cultural behavior, skin pigmentation, and gene-environment interaction contribute to reduced serum 25-hydroxyvitamin D [25(OH)D] concentrations.²

Historically, vitamin D and sunlight exposure were used to treat TB in the preantibiotic era, and there is increasing evidence from *in vitro* studies that vitamin D enhances antimycobacterial immunity.³ A recent study conducted among adult TB contacts found that 94% of recruits were vitamin D insufficient [serum 25(OH)D <75 nmol/L] and that a single, oral 2.5 mg dose of vitamin D significantly enhanced their antimycobacterial immunity *in vitro*.⁴

Vitamin D in humans is obtained from 2 sources: sunlight exposure and ingestion of cholecalciferol (found in fish, meat, and vitamin D-fortified foods). A vegetarian diet increases the risk of a person being vitamin D deficient, whereas lack of sunlight (especially in Britain from November to April), application of suntan lotion, pigmented skin, and the wearing of long-sleeved clothes reduce cutaneous synthesis.⁵

We were unable to find recent published data on vitamin D deficiency in UK pediatric TB patients. We therefore set out to determine whether vitamin D deficiency was common among children in our clinic with active or latent TB, to evaluate the effects of gender, age, ethnicity, and seasonality on serum 25-hydroxyvitamin D concentrations, and to determine whether alkaline phosphatase, commonly used as a surrogate marker of vitamin D deficiency, was a predictive factor.

MATERIALS AND METHODS

We undertook a retrospective case notes review of all patients attending the pediatric TB clinic between June 2004 and June 2006 with a diagnosis of either active TB or latent TB infection (LTBI). We collected demographic details and serum 25-hydroxyvitamin D concentrations and alkaline phosphatase concentrations in serum, measured at the time of diagnosis and before commencing TB treatment. Analysis of serum 25-hydroxyvitamin D was by tandem mass spectrometry. Statistical analysis was completed with the Mann-Whitney and unpaired *t* tests using Graph Pad Prism v 4.0.

RESULTS

We identified 67 patients, and serum 25-hydroxyvitamin D results were available for 64 patients. The median age of children was 8.4 years (range, 0.1–17 years) and 34 (53%) were male (Table 1). Most patients were from ethnic minority groups of whom 24 (38%) were black African and 35 (55%) were South Asian.

Eighty-six percent ($n = 55$) of children were either serum 25-hydroxyvitamin D deficient [serum 25(OH)D <20 nmol/L] or insufficient [serum 25(OH)D <75 nmol/L].⁶ Only 1 child with active TB had normal vitamin D values. Children with active TB presenting between November and April had significantly lower serum 25-hydroxyvitamin D concentrations (mean, 21.5 nmol/L; interquartile range, 11–32 nmol/L) than those presenting between May and October; (mean, 41.7 nmol/L; interquartile range, 18–54 nmol/L, $P = 0.02$). However, there was no effect of seasonality on LTBI ($P = 0.1$). Children older than 10 years of age had significantly lower serum 25-hydroxyvitamin D concentrations (median, 24 nmol/L; interquartile range, 15–35 nmol/L) than children younger than 1 year of age (median, 78 nmol/L; interquartile range, 42–101 nmol/L, $P < 0.002$).

Gender, TB status, and ethnicity had no significant effect on vitamin D concentration and there was no correlation between

TABLE 1. Gender, Ethnicity, Age, and Vitamin D Measurements of Study Subjects

Variable	All Patients	Active TB	LTBI
Number (%)	64	26 (41)	38 (59)
Median age in yrs (range)	8.5 (0.1–17)	12.7 (2.5–17)	4.7 (0.1–16.4)
Male sex (%)	34 (53)	14 (53)	20 (52)
Ethnic group*			
Black African (%)	24 (37)	6 (23)	18 (47)
South Asian (%)	35 (55)	17 (65)	18 (47)
Other (%)	2 (3)	1 (4)	1 (3)
Unknown (%)	3 (5)	2 (8)	1 (3)
Median serum vitamin D (range) (nmol/L)	26.5 (2–132)	22.5 (2–80)	28 (8–132)
Vitamin D deficient (%) (<20 nmol/L)	24 (37.5)	12 (46)	12 (32)
Vitamin D insufficient (%) (<75 nmol/L)	31 (48.5)	13 (50)	18 (47)
Vitamin D replete (%)	9 (14)	1 (4)	8 (21)

*South Asians included Indians, Pakistanis, Afghans, and Nepalis.

⁶“Other” ethnicity comprised 1 Caucasian and 1 Chinese patient, both of whom were vitamin D replete.

alkaline phosphatase measurements in serum and serum 25-hydroxyvitamin D concentrations ($r = 0.18$), data not shown. Of the 64 patients included in the analysis, only 1 patient was found to have an elevated alkaline phosphatase value.

DISCUSSION

We have found a high percentage of vitamin D deficiency and insufficiency in children attending our pediatric TB clinic, most marked in children older than 10 years of age and with a significant seasonal variation. Environment, sociocultural factors, and host genetics are all likely to be contributors to this finding. Although we acknowledge that the principal limitation of our study is the lack of an age and ethnically matched control group without a history of TB infection or disease, to our knowledge, there is no recent published data on the prevalence of asymptomatic vitamin D deficiency in UK children.

Our clinic population is representative of many UK TB clinics, in which South Asian and black African migrant groups predominate and where vegetarian diet, clothing, and dark skin all contribute to reduced serum 25-hydroxyvitamin D concentrations. Many Hindus and Muslims in our population wear long clothing that reduces their exposure to sunlight. It may also be that the limited provision of safe, outdoor play facilities in dense, inner city environments, together with more sedentary lifestyles contribute to inadequate vitamin D stores by limiting sunlight exposure. Although we did not collect data on infant-feeding practices, the most likely explanation for the higher levels of vitamin D seen in infants was bottle feeding with supplemented milk formula.

Between November and April in the United Kingdom there is usually insufficient sunlight to allow development of adequate vitamin D reserves, and this may explain the seasonal variation in vitamin D status seen in our study and is consistent with the finding that TB notification rates are higher in the summer months, possibly because of a lag period between depletion of vitamin D stores and activation of LTBI.⁷

Although there are no published studies measuring the likely contribution of low serum 25-hydroxyvitamin D concentrations on conversion rates of LTBI to active TB, recent data not only support a role for vitamin D in protection from TB but also lend biologic plausibility to the historic treatment of TB with sunlight. The active form of vitamin D (1,25-dihydroxyvitamin D₃) boosts concentrations of the antimicrobial peptide LL37 (cathelicidin) in human neutrophils³ and incubating infected human monocyte cultures with increasing doses of vitamin D restricts mycobacterial growth.⁸ Furthermore, it has been shown that a single oral dose of vitamin D restricts the growth of mycobacteria in an in vitro whole blood model of infection.⁴ Thus sunlight (and dietary supplementation), by raising the circulating levels of vitamin D, might increase the capacity of monocytes and macrophages to kill microbes and, specifically, mycobacteria.

Finally, our results show that a raised alkaline phosphatase value is a poor indicator of vitamin D deficiency in children. Dependence on this test would significantly underestimate the level of deficiency in a vulnerable population. Given the increasing evidence base to support a role for vitamin D in the innate immune response to mycobacteria, the very high levels of vitamin D deficiency that we report in this group of children is of concern. Further studies of vitamin D status among healthy children of the same and different ethnic background need to be completed, and prospective randomized controlled trials are needed to validate our observations and to establish whether giving adjunctive vitamin D to children with TB is of benefit.

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THE ROLE OF HAEMOPHILUS INFLUENZAE TYPE b IN FATAL COMMUNITY-ACQUIRED PNEUMONIA IN CHINESE CHILDREN

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Abstract: To analyze the role of *Haemophilus influenzae* type b in children's death resulting from severe pneumonia conventional PCR, Southern blotting and in situ PCR were evaluated for the detection of Hib in 202 paraffin-embedded lung tissue autopsy samples from children with fatal outcome of pneumonia in China during the period of 1953–2002. The results indicate that Hib was a cause of fatal pneumonia in 36 (17.8%) of 202 samples.

Key Words: community-acquired pneumonia, fatal outcome, etiology, *Haemophilus influenzae* type b, in situ PCR, China

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In China, community-acquired pneumonia (CAP) remains the major cause of children's morbidity and mortality. *Streptococcus pneumoniae* and *Haemophilus influenzae* type b (Hib) are responsible for most CAP cases.^{1,2} Because most patients with CAP are likely to be treated with antibiotics before visiting the hospital, the isolation rates of Hib are very low in China. Furthermore, the role of these 2 pathogens in childhood CAP in China, especially in severe or fatal CAP, has not been clarified. Such information is important in the development of strategies for vaccine prophylaxis and antibiotic therapy.

Most routine approaches for the diagnosis of Hib in pneumonia such as blood culture and serology are not sensitive.³ This is particularly important when specimens are collected from nonsterile sites (via nasopharyngeal or throat swabs) because colonization of these organisms is common in children. Lung puncture for culture is not accepted by patients and their parents in China. Therefore,

specific diagnostic techniques to the etiologic agents of CAP, particularly in severe or fatal cases, are needed.

The present study was performed to analyze the role of Hib as the causative agent in the fatal outcome of severe pneumonia among Chinese children. To achieve this objective, conventional polymerase chain reaction (PCR), Southern blotting, and in situ PCR (ISPCR) were applied for the detection of Hib to paraffin-embedded lung tissue autopsy samples from children with fatal outcome of CAP in China in the period of 1953–2002.

METHODS

A total of 202 cases of children ranging from 1 month to 5 years old (≤ 2 years, $n = 172$; > 2 years, $n = 30$) who died of CAP with available paraffin-embedded lung autopsy tissues were included in this study. Among these 202 samples, 116 samples were collected in the period of 1953–1969, while 86 samples were collected from 1980 to 2002. A group of 40 cases of paraffin-embedded autopsy lung tissue from age-matched children without pneumonia and other infections was also included in this study to serve as control. The samples were randomly selected from the collection at Beijing Children's Hospital.

Bacterial DNA from paraffin-embedded lung tissues was extracted as follows: 10- μ m thick tissue sections were digested with 0.5 mg/mL proteinase K (SERVA, Germany), and DNA was isolated via phenol/chloroform extraction.

Two primers, HibFor, 5'-CCT CGC AAT GCA GTT TAT GGT CC-3' and HibRev, 5'-AAG CGG GAA TTT GAT ACC TGA TGC-3', for the gene involved in Hib capsule synthesis were designed using the program OLIGO. The amplification program was performed as previously described.⁴

The procedures involved in DNA labeling by digoxigenin, Southern blotting, and immunologic detection were performed according to the protocols provided by the manufacturer (Roche Corp., Germany).

ISPCR was performed as previously described.^{5–6} The paraffin-embedded mice lung tissues infected by Hib fixed on the glass slides were used as positive controls. PCR mixtures lacking *Taq* DNA polymerase or primers, normal lung tissues were used as negative controls. The statistical methods used in this study include independent-samples *t* test and χ^2 test, with $P < 0.05$ considered as significant for all comparisons.

RESULTS

In paraffin-embedded lung tissue samples of 202 children who died of pneumonia conventional PCR, Southern blotting and ISPCR revealed the presence of Hib in 2 (1.0%), 28 (13.9%), and 36 (17.8%) samples, respectively. Using ISPCR, Hib was found to be distributed diffusely in the lung tissue, especially in areas with hyperemia or hemorrhage. A total of 13.9% of the samples (28 of 202) were found to be positive by both Southern blotting and ISPCR. No Hib was detected in similar specimens from the control group by any of the 3 methods. Hib was identified in 18 of 116 (15.5%) samples collected from 1953 to 1969 and in 18 of 86 (20.9%) samples collected from 1980 to 2002 ($\chi^2 = 0.988$, $P > 0.05$) (Fig. 1). *H. influenzae* type b was detected in 32 of 172 (18.6%) lung tissue samples of children who died younger than 2 years old, in 4 of 30 (13.3%) samples of children who died older than 2 years old ($\chi^2 = 0.485$, $P > 0.05$).

All available chest radiographs confirmed the presence of alveolar consolidation, indicating probable bacterial infection. On the other hand, no pneumonia had been diagnosed pathologically in all patients from the control group.

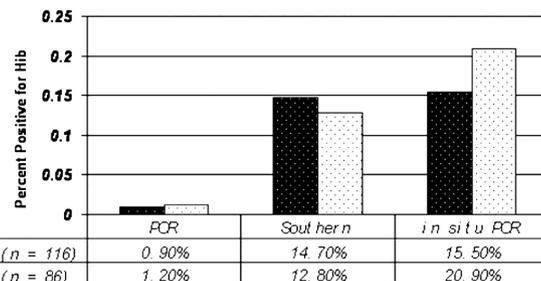


FIGURE 1. Presence of Hib DNA in paraffin-embedded lung tissue autopsy samples of children who died of CAP in China (1953–2002).

DISCUSSION

In this study, on fatal cases of childhood CAP, the Hib was considered the causative agent in 17.8% of cases. All samples selected in this study group exhibited typical pathologic findings for pneumonia, including consolidation with pulmonary infiltrate and intense polymorphonuclear leukocytes in bronchioles and adjacent alveoli, with or without tissue necrosis. These results provide strong evidence that Hib is a significant pathogen of childhood pneumonia in China.

The level of Hib detection through molecular methods is higher than that through the microbiologic methods. Most patients with CAP are likely to be treated with antibiotics before visiting the hospital, which could affect the etiologic diagnosis. Molecular techniques can detect the presence of Hib DNA from killed or damaged bacterial cells as it occurs after antibiotic treatment.

All 3 methods used in this study were highly specific for Hib detection. However, their respective sensitivities varied. ISPCR expressed the highest sensitivity (36 of 202 samples). This could be because of the loss of bacterial DNA during extensive extraction and purification procedures for conventional PCR. Additionally, signal amplification in the detection of amplified DNA in ISPCR can further enhance the sensitivity of the method.

Moreover, ISPCR provides valuable information on the location of Hib in tissues and its correlation with histopathologic changes.^{6–8} In this study, Hib localized to lung tissues and mainly in the pulmonary alveolus, and in or around the dilated and congested vessels, but not inside the macrophages or lung cells.

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SEVERE CLOSTRIDIUM DIFFICILE—ASSOCIATED DISEASE IN CHILDREN

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Abstract: Three cases of *Clostridium difficile*-associated disease in children were detected within a short time interval. Intensive therapy was required in 2 cases with colectomy in one of them. One of the severe cases was community-acquired. Two patients had underlying diseases (Hirschsprung disease, Down syndrome) and also tested positive for enteric viruses (rotavirus, calicivirus).

Key Words: *Clostridium difficile*, child, pseudomembranous colitis, ribotyping

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Clostridium difficile is considered an important pathogen in hospitalized patients aged >65 years and receiving antibiotic therapy; its role in children is still controversial.¹ In several studies, high carriage rates were reported in asymptomatic neonates and young children^{2,3} and at present testing for *C. difficile* is rarely performed in children <2 years of age. Nevertheless, *C. difficile* can cause gastrointestinal symptoms in neonates and children,^{1,4} and is an important pathogen in immunocompromised young patients⁵ and in those with Crohn's or Hirschsprung disease.⁶

Recently, attention has been paid to the emergence and spread of a highly virulent strain, BI/NAP1/027, causing increased mortality and disease severity, although the highest prevalence was observed in patients >65 years of age.⁷ Increased incidence has also been detected in patients previously considered at low risk, especially children and adolescents. In a report by Chernak et al⁸ 11 of the 23 patients with community-acquired *C. difficile* disease were <18 years of age.

We report 3 cases of *C. difficile* infection in children detected within 2 months (from October to December 2006) at the University Medical Centre Ljubljana, Slovenia (summarized in Table 1, <http://links.lww.com/A479>). Two of the cases were severe requiring intensive or surgical treatment.⁷ Until now, *C. difficile* infections of such severity have not been seen in pediatric patients at our institution and *C. difficile* was not frequently sought as a cause of diarrhea in children.

CASE REPORTS

Case 1. A 13-year-old girl was admitted to the Department of Infectious Diseases with a 2-day history of fever, vomiting, and diarrhea resulting in syncope. She had recently completed a 5-day course of amoxicillin-clavulanic acid for skin infection. The patient developed hypovolemic shock requiring intensive care unit (ICU) admission, fluid resuscitation, and inotrope support. The laboratory was characterized by elevated C-reactive protein (330 mg/L) and leukocytosis ($27 \times 10^9/L$) with increased immature cells. *C. difficile* infection was suspected but because the latex test for *C. difficile* A toxin (Becton Dickinson) was negative, the patient was given cefotaxime for presumed sepsis on admission to the ICU. Subsequently, stool tested with enzyme-linked immunosorbent assay (ELISA) for A/B toxin (Meridian) was positive. Because blood cultures remained negative, cefotaxime was substituted with oral and due to vomiting with parenteral metronidazole. Because the patient did not improve adequately, oral and later rectal vancomycin was added to the therapy. Colonoscopy demonstrated pseudomembranous colitis predominantly affecting the distal colon. *C. difficile* was grown in culture from the stool and from colonic aspirate. The patient gradually recovered and completed 10 days of vancomycin (including 4 days given rectally) and 14 days of metronidazole treatment. The disease course was complicated with pleural effusion requiring evacuation. The pleural fluid was classified as transudate and was probably due to hypoalbuminemia. No further investigations were performed because no fluid reaccumulated. At 2 months post-hospital discharge, the patient was considered cured and no sequelae were present.

Case 2. An 8-month-old boy was hospitalized with a 24-hour history of fever, vomiting, and diarrhea. He had had surgery for Hirschsprung disease at the age of 3 months and was last hospitalized at the Department for Pediatric Surgery because of constipation problems 1 month earlier when he received penicillin orally for 10 days for perianal skin infection. His 3-year-old sister had contact with horses and developed diarrhea several days before him. The boy had nonbloody diarrhea. The course of disease was characterized by high fever and elevated C-reactive protein (214 mg/L). Stool culture was negative for *Salmonella*, *Shigella*, *Campylobacter*, and *Escherichia coli* and a latex test was negative for *C. difficile* A toxin. Subsequently, stool sample tested positive for the presence of *C. difficile* A/B toxin by ELISA and *C. difficile* was isolated in stool culture. Calicivirus was detected by ELISA in a stool sample obtained on day 3 of hospitalization (although it had tested negative on day 1). The course of disease was relatively mild and the patient improved with oral metronidazole therapy he received for 7 days.

Case 3. A 10-year-old girl with Down syndrome was admitted to the pediatric ICU after complete surgical correction of Fallot tetralogy. In the early postoperative period she developed fever and received teicoplanin and ceftazidime. Despite antibiotic treatment, the patient's condition deteriorated and, subsequently, she was given imipenem and fluconazole. The patient required intensive therapy including peritoneal dialysis for renal failure. Eight days after surgery, she developed a distended, painful abdomen followed by watery and later bloody diarrhea, peritonitis, and paralytic ileus

resulting in multiorgan failure and shock. After insertion of peritoneal drains, 500–800 mL of odorless, brownish fluid was collected daily. The stool tested positive for *C. difficile* A/B toxin by ELISA and for rotavirus by ELISA and electron microscopy. *C. difficile* toxin was also positive in peritoneal fluid. Despite oral vancomycin, the patient's condition further deteriorated and oral metronidazole and rectal (because of paralytic ileus) vancomycin were added to therapy. Sigmoidoscopy demonstrated pseudomembranous colitis. Because intestinal perforation was suspected, laparotomy was performed 52 days after heart surgery, demonstrating necrotic transverse colon with several abdominal abscesses and numerous perforations of the terminal ileum. Peritoneal lavage with terminal ileostomy placement and colectomy was performed. The patient received 21 days of vancomycin and 33 days of metronidazole treatment and was discharged from the hospital 93 days after heart surgery in a stable clinical condition.

DISCUSSION

An unusual cluster of 3 *C. difficile* infections in pediatric patients is described. All 3 cases were positive for *C. difficile* toxin A, but strains were isolated only from cases 1 and 2 in the diagnostic laboratory where they were frozen and subsequently made available for molecular characterization. Both strains were grouped in the toxinotype 0⁹ and correspondingly had no genes for binary toxin when tested by PCR method.¹⁰ Both strains were ribotyped as described by Stubbs et al.¹¹ Epidemiologic association of case 3 with a hospitalized case (case 2) could not be ruled out because no isolate was available for case 3. Still, in case 1 the strain was community-acquired and was in ribotype profile different from the strain from case 2.

Two patients (case 2 and 3) had underlying conditions previously reported to be associated with *C. difficile* disease (Hirschsprung disease and Down syndrome) and both had previously been hospitalized. The source of infection in case 1 was less obvious. There was indirect contact with a hospital environment because the patient's mother worked in the hospital as a nurse. She has not been tested. Also, the girl had regular contact with a horse. The animal tested negative for *C. difficile*, but testing was performed more than 2 months after the girl's disease. The horse was asymptomatic at the time of sampling, but occasionally received antibiotics because of various problems due to advanced age. Indirect contact with a horse was also present in case 2 via the patient's sister who developed a mild diarrheal episode (with no stool examination) before her brother. The second horse also tested negative for *C. difficile*. The animals could therefore not be proven as sources of infection in our cases but horses have been increasingly recognized as important hosts for *C. difficile*, as well as pigs and calves.¹²

Apart from the above described cases, at the Department of Infectious Diseases *C. difficile* toxin was positive in 6 additional symptomatic children with milder disease in the period from November 2006 to May 2007. Four of these patients also tested positive for enteric viruses (2 for rotavirus and 2 for calicivirus), one had simultaneous pneumococcal bacteremia, and one had adenoviral upper respiratory tract infection. All patients had previously been treated with antibiotics. When comparing these patients with the 3 severe cases, significantly lower C-reactive protein (CRP) values (mean value 37 versus 259 mg/L) and white blood cell (WBC) counts (14.4 versus $24.3 \times 10^9/L$) were seen in mild cases and in all severe cases a left shift was observed.

Reports from North America suggest that the gradual emergence of *C. difficile* in populations previously considered at low risk seems to correlate with the increased incidence and severity of *C. difficile* infections reported in recent years.^{8,13} Although there is no national *C. difficile* surveillance system in Slovenia, the situation

could be different from that in the United States and in some European countries in that epidemic type BI/NAP1/027 has so far not been detected and none of the hospitals had reported increased infection rates (Rupnik, unpublished data). In other countries, fulminant community-acquired cases requiring hospitalization were associated with variant toxinotypes (other than toxinotype 0) and producing binary toxin, but not necessarily of the type BI/NAP1/027.^{8,14,15} In contrast, strains isolated in this study were nonvariant toxinotypes 0, indicating again that such strains can cause severe disease.

When caring for patients with gastrointestinal symptoms, especially when high WBC counts and/or elevated CRP are present, clinicians should consider *C. difficile* infection in the differential diagnosis. This is particularly important in countries that have not yet experienced epidemics with the new hypervirulent type 027 and where the physicians caring for patients at low risk for *C. difficile* infection are not aware of this problem. In some cases, as exemplified by this report, because of low sensitivity the rapid microbiologic tests give false negative results, which is one of the well known problems in *C. difficile* diagnostics. Therefore, not only rapid *C. difficile* specific tests but also tests with high sensitivity and close cooperation between clinicians and microbiologists are needed to ensure early recognition and treatment of this severe disease.

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ACYCLOVIR-RESISTANT CHRONIC VERRUCCIOUS VACCINE STRAIN VARICELLA IN A PATIENT WITH NEUROBLASTOMA

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Abstract: A 21-month-old girl with neuroblastoma developed chronic verrucous Oka strain varicella-zoster infection during chemotherapy. Virus isolated from the patient demonstrated high-level acyclovir resistance, and its thymidine kinase had no in vitro enzymatic activity. After foscarnet therapy, she underwent stem cell transplantation without varicella reactivation. This is only the second reported case of resistant varicella zoster virus caused by Oka strain virus.

Key Words: varicella, Oka, acyclovir, resistance, immunosuppressed, neuroblastoma

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UAB Institutional Review Board approved informed consent was obtained from the patient's family before compilation of this report. No contributors to the manuscript have any proprietary, financial, professional, or other personal interest of any nature or kind in any product, service, or company that could be construed as influencing the material, position, or review of the manuscript.

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The varicella zoster virus vaccine strain Oka (V-Oka) is highly attenuated and can prevent severe disease associated with varicella infection. However, in rare instances it can cause disseminated infections in immunosuppressed patients that require prompt and prolonged therapy.

CASE REPORT

A 21-month-old girl was diagnosed with stage IV neuroblastoma. She had received varicella immunization in her left thigh

approximately 1 week before diagnosis and had no other known history of varicella exposure. Her chemotherapy plan consisted of a combination of 4 courses of cyclophosphamide, adriamycin, and vincristine (CAV) and 2 courses of cisplatin and etoposide (CDDP-E) to be followed by autologous stem cell transplant.

Four weeks into chemotherapy, during a period of leukocyte recovery, the patient developed 2 erythematous, umbilicated papules on her right finger and right lower abdomen distant from the immunization site. In the ensuing week, these lesions evolved into vesicular patches, primarily involving her hands, right leg, and abdomen. Serum anti-varicella IgG was positive at this time and polymerase chain reaction (PCR) of lesion scrapings was positive for varicella zoster virus (VZV). Intravenous acyclovir (IV ACV) was started at 60 mg/kg/d and the lesions improved after 1 week of IV therapy. Over the next 2 weeks, therapy was changed to oral ACV therapy at 2 separate times (90 mg/kg/d and later 30 mg/kg/d), but readmission was required for IV therapy within 2 days each time because of worsening lesions and missed oral doses as a result of emesis. Finally, IV ACV (60–70 mg/kg/d) was restarted and continued for 3 additional weeks. During the 5 weeks of IV and oral ACV therapy, the lesions became verrucous and dusky but never healed completely (Fig. 1, <http://links.lww.com/A481>), and new vesicles continued to erupt intermittently, primarily on her right leg and lower abdomen. VZV was isolated from a newly erupted vesicle, and this isolate exhibited high level resistance in vitro to both ACV and penciclovir (Table 1). Therapy was changed to IV foscarnet (165 mg/kg/d) and continued for a total of 4 months with intravenous immunoglobulin (1 g/kg/dose) given approximately every 2–4 weeks. After 4 weeks of foscarnet therapy, repeated cultures of skin scrapings were culture negative but remained VZV PCR positive.

Eight weeks after starting foscarnet, the patient developed lethargy, fatigue, photophobia, and conjunctivitis. Lumbar puncture revealed leukocytosis (protein 166 mg/dL, 5 WBC per HPF, 92% lymphocytes), and PCR of cerebrospinal fluid (CSF) was positive for VZV, though live virus was never isolated from the CSF. The 6th planned cycle of chemotherapy was withheld, and she received 1 dose of intravenous varicella immunoglobulin (VariZig; Cangene Corporation, Winnipeg, Canada) on an investigational compassionate use basis. Additional doses of VariZig were withheld because of acute renal insufficiency and proteinuria. The patient's symptoms slowly improved, but cerebrospinal fluid obtained by serial lumbar puncture remained positive for VZV by PCR despite foscarnet therapy for 10 weeks. All virus cultures of the CSF were negative. She exhibited moderate developmental delay neurologically, possibly because of CNS varicella or anesthesia-related respiratory arrest that occurred early in therapy.

After completing induction chemotherapy and consolidative radiotherapy, she remained in remission on 13-cis retinoic acid for

TABLE 1. Sensitivity of the Oka and Isolate 6/06/2 to Selected Antiviral Drugs

Drug	EC ₅₀ *		Fold-Resistance
	Oka	6/06/2	
Ganciclovir	11 ± 5	47 ± 13.2	4.3
Cidofovir	0.56 ± 0.01	0.7 ± 0.14	1.2
Acyclovir	5.3 ± 2.2	>100 ± 0	>19
Penciclovir	15 ± 0.07	>100 ± 0	>6.7
Foscarnet	58 ± 7.3	60 ± 8.4	1.0

*EC₅₀ values are the drug concentrations required to reduce plaque number by 50% and are given in units of micromolar with the standard deviations shown.

3 months, but neuroblastoma recurred. She underwent reinduction chemotherapy with ifosfamide, etoposide, cyclophosphamide, and topotecan with good response and ultimately had a successful autologous stem cell transplant utilizing VZV PCR negative peripheral blood stem cells. She remained in good health until 6 months post-transplantation when neuroblastoma again relapsed. After a trial of palliative therapy, the child died from complications of her disease. She never demonstrated clinical evidence of varicella relapse after transplant.

MATERIALS AND METHODS

Virus Purification and Characterization. Varicella-zoster strain Oka was obtained from American Type Culture Collection (Manassas, VA) and passaged as described previously.¹ The clinical isolate 6/06/2 was obtained from a swab of unroofed lesions from the patient. This isolate was placed in 25 cm² flasks with freshly trypsinized primary human foreskin fibroblast cells. Infected cells were passaged and expanded to produce virus stocks for subsequent studies. Susceptibility to antiviral drugs was determined by plaque reduction assay with methods described previously.² Drugs were obtained from the NIAID or purchased from Sigma Aldrich (St. Louis, MO). Cidofovir was a gift from Mick Hitchcock at Gilead Sciences (Foster City, CA).

Viral DNA was extracted from cells infected with Oka or 06/06/2 by standard methods. The TK gene was amplified using primers 5'-CAC CAT GTC AAC GGA TAA AAC CGA TGT AA-3' and 5'-GGA AGT GTT GTC CTG AAC GGC ATT-3'; ORF62 was amplified using primers 5'-AAG TTG GCA AAC GCA GTC-3' and 5'-ATT ACT GTC GAC CCG AGA CC-3' and ORF28 was amplified using primers 5'-ATG TCA TCG TTT CAA TTT TTG G-3' and 5'-TTA ACT TTG ATG GAG AAT TGC TTT TGG-3'. Resulting PCR products were sequenced using a set of internal primers to sequence both strands of the DNA. The TK open reading frames from both strains were cloned into the pET151d bacterial expression vector (Invitrogen, Carlsbad CA) and the open reading frames were completely sequenced. The enzymes from both strains of VZV were expressed in *Escherichia coli* BL21 cells and were purified and assayed by methods described previously.³ Briefly, enzymatic activity was determined in a luciferase assay using thymidine as a substrate and used ATP consumption as a marker for enzymatic activity.

RESULTS

Susceptibility to Antiviral Drugs. The clinical isolate (6/06/2) was cultured, amplified, and its susceptibility to a set of representative antiviral drugs was determined by standard plaque assay using Oka as a fully susceptible control virus. The 6/06/2 isolate was fully susceptible to both foscarnet and cidofovir, whereas it was modestly resistant to ganciclovir (GCV) and highly resistant to both ACV and penciclovir (PCV) (Table 1). These results seem to be consistent with the clinical course including the inability of ACV to limit the infection.

Molecular Characterization of 06/06/2. Significant differences were observed in drug sensitivity between the clinical isolate and the Oka strain. Initial studies sought to confirm that the clinical specimen was derived from the vaccine strain. A previous report identified a polymorphism diagnostic of the vaccine strain, which is used by the vaccine manufacturer to confirm vaccine strain identity.⁴ This region of the clinical isolate was sequenced and did contain a *Sma*I site characteristic of the Oka strain, indicating that the patient's isolate was derived from the vaccine virus. To characterize the mutations that the strain acquired to become resistant to the drugs, the coding

regions of the TK and polymerase genes were completely sequenced. A single mutation was observed in 06/06/2, resulting in a T256A mutation in the amino acid sequence of the TK. Seven additional mutations were also identified in the coding sequence of the viral DNA polymerase. Amino acid mutations identified were Q452R in region IV, C566Y and I593V in D-region C, and L703S and N887S in regions II and I, respectively.⁵ Two additional mutations, V122I and F182R, were also observed in the amino terminus of the polymerase. Mutations that confer resistance to ACV have been identified previously in the carboxyl terminus of the TK open reading frame near the T256A mutation in this isolate. To confirm that this mutation contributed to drug resistance, the open reading frames from Oka and from 06/06/2 were cloned into a bacterial expression vector, expressed in bacteria, and purified. The enzyme expressed from the Oka open reading frame was highly active with thymidine as a substrate and had a V_{max} of 6.8 $\mu\text{M min}^{-1} \mu\text{g}^{-1}$, whereas the enzyme expressed from the 06/06/2 ORF did not exhibit detectable activity ($<0.25 \mu\text{M min}^{-1} \mu\text{g}^{-1}$). Thus this mutation is likely sufficient to explain the observed resistance to ACV, PCV, and GCV that each require phosphorylation by this viral enzyme. The additional mutations in the polymerase gene are also similar to those reported in drug resistant strains, but were not investigated further. The TK mutation in this isolate is likely sufficient for the high level of antiviral resistance observed for this virus.

DISCUSSION

This case represents a second report describing chronic disseminated varicella disease caused by acyclovir-resistant Oka strain VZV, but it is the first to document recovery of a replication-competent Oka strain viral isolate from a patient. The only other published report of acyclovir-resistant Oka strain VZV also occurred in a 1-year-old child who began chemotherapy for neuroblastoma shortly after administration of the varicella vaccine.⁴ In both cases, the hyperkeratotic, verrucous appearance of the patient's rash is consistent with the well-described clinical entity of chronic, ACV-resistant wild-type VZV infection in immunocompromised patients.⁶⁻⁹ The clinical appearance of this rash should thus alert the clinician to the possibility of antiviral-resistant VZV, even if disease is believed to be caused by Oka strain varicella.

This case is the first report of a live viral isolate derived from Oka strain recovered and characterized from a patient with clinical suspicion for antiviral resistance, demonstrating *in vivo* viral replication during ACV therapy. In prior reports, DNA was isolated from clinical lesions and antiviral sensitivity was characterized based on the DNA sequences. Our inability to recover infectious virions from skin lesions after initiation of foscarnet therapy suggests that foscarnet was able to limit viral replication *in vivo*, although the patient failed to achieve complete resolution of symptoms until cessation of chemotherapy and reconstitution of the immune system.

The Oka strain varicella vaccine is highly attenuated and is generally accepted as safe in immune-competent individuals^{10,11}; however, its use is restricted in those with impaired cellular immunity or in close family contacts of such patients.^{12,13} Current recommendations state to withhold vaccination in patients with leukemia, lymphoma, or other malignancies in remission until at least 3 months after cessation of chemotherapy.¹² These 2 cases of resistant vaccine strain varicella in patients with malignancy argue the need for particular attention regarding status of varicella vaccination history at the time of cancer diagnosis in pediatric patients. Some experts recommend initiating prophylactic oral ACV (80 mg/kg/d) if an immunosuppressive event occurs within 4-6 weeks of vaccination and continuing ACV prophylaxis until completion of chemotherapy or the immunosuppressive event. Prompt treatment with

intravenous ACV should be initiated at the first signs of infection to avoid development of resistance, even in Oka strain VZV. This virus likely became resistant in an environment of impaired immunity and inconsistent ACV dosing because of intolerance of oral therapy. The role of IVIG and varicella immunoglobulin (VariZig) in this setting is less clear.

Because of the grim prognosis of stage IV neuroblastoma, autologous transplantation is generally considered front-line therapy. The chance of wild-type varicella reactivation postautologous transplant may be as high as 48% in high-risk patients, although disseminated noncutaneous disease is rare.¹⁴ The morbidity of CNS reactivation in the immunocompromised patient can be particularly severe, potentially leading to myelitis, encephalitis, and arteritis.¹⁵ These factors were considered heavily in the treatment of this patient for whom the risk of both varicella reactivation and neuroblastoma relapse were high. Our experience and that of Levin et al⁴ is that autologous transplantation may be feasible in similar cases after sufficient time has elapsed to permit immune reconstitution.

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POLYOMA VIRUS HEMORRHAGIC CYSTITIS IN AN OTHERWISE NORMAL CHILD

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Abstract: We describe a case of polyoma virus hemorrhagic cystitis in a nonimmunosuppressed child. Polyoma virus infection was suspected because of abnormal urine cytology. Polyoma virus cystitis in nonimmunosuppressed children is self-limited, resolving spontaneously within 2 weeks.

Key Words: polyoma virus, hemorrhagic cystitis

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Acute hemorrhagic cystitis is a pediatric problem that has multiple etiologies.¹ A bacterial etiology can be identified by urine culture. A viral etiology, however, requires special tests.² We present a case of viral hemorrhagic cystitis where the diagnosis of polyoma virus was suspected because of abnormal cytology.

CASE PRESENTATION

A 3-year-old circumcised boy presented to his pediatrician with a 2-day history of pink urine, dysuria, increased frequency, and urgency. He had no prior medical problems. The physical examination was normal. Urinalysis revealed blood without casts; leukocyte esterase and nitrites were negative. Urine culture was sterile. An abdominal ultrasound examination was performed and showed a questionable area of bladder wall thickening; no urinary stones were identified. Urinary cytology showed a cluster of uroepithelial cells with smudgy chromatin, suggesting polyoma viral cytopathic effect. Confirmatory immunohistochemistry staining for the SV40 antigen was positive (Fig. 1). The patient was referred to the infectious disease clinic. On day 11 of symptoms, he complained only of dysuria. Urinalysis showed RBCs without casts. Urine cytology demonstrated polyoma cytopathic effect, which was confirmed by immunohistochemistry staining. Concurrent bacterial culture was negative. Urine viral culture for adenovirus and cytomegalovirus was negative. Over the next week, his dysuria resolved and follow-up studies (urinalysis, ultrasound, cytology, and immunohistochemistry) were negative. The patient has remained well during the last 5 years.

DISCUSSION

Causes of hemorrhagic cystitis include bacteria (especially *Escherichia coli*), viruses [ie, adenovirus, polyoma (BK) virus, influenza A, herpes simplex], chemical toxins, bleeding disorders,

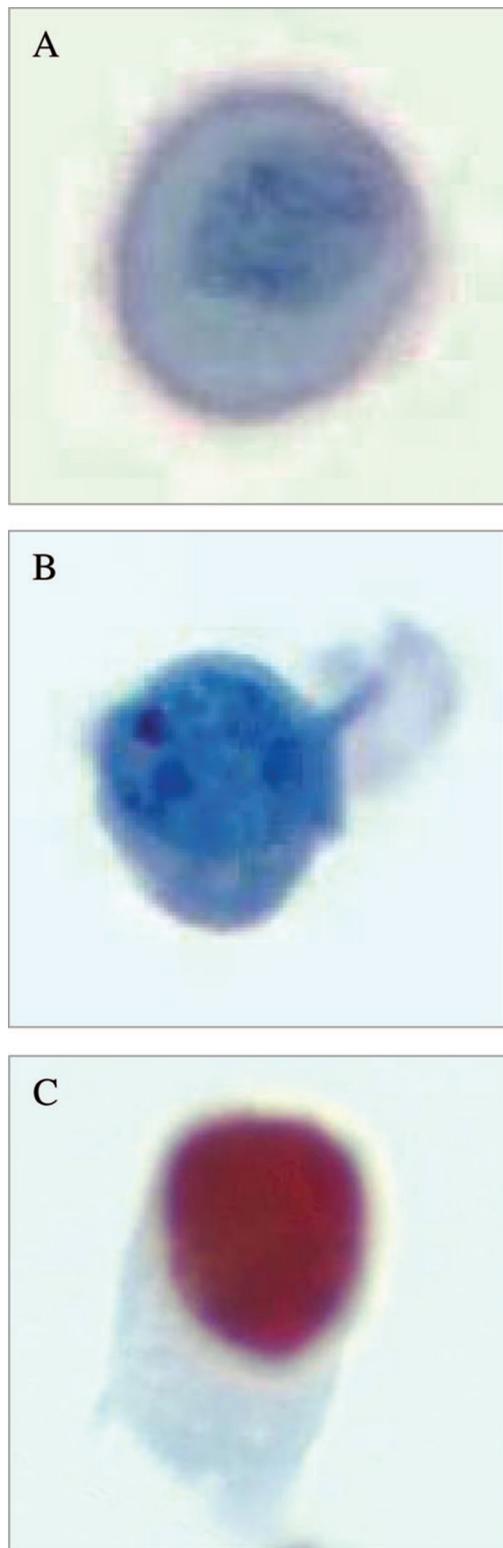


FIGURE 1. Normal urothelial cell with homogeneous nucleus surrounded by abundant nucleus (A, PAP Stain 100 \times). Polyoma viral effect with increased nuclear size, clumped chromatin, prominent nucleoli, and decreased cytoplasm (B, PAP Stain 100 \times). Positive red nuclear staining for SV40 polyoma virus (C, SV40 immunohistochemistry stain 100 \times).

radiation, and idiopathic.² In a study of 19 pediatric patients with hemorrhagic cystitis,³ adenoviruses (types 7 or 11) were recovered from 8, *E. coli* from 2, and the cause was not defined in 9 patients. The gross hematuria ranged from 3 to 15 days. Cytology was not performed.

The polyoma viruses include BK virus (associated with nephropathy and/or cystitis in immunodeficient patients), JC virus (associated with progressive multifocal leukoencephalopathy in immunodeficient patients), and simian virus (SV) 40 (a virus from the rhesus macaque that contaminated polio virus vaccines in the 1960s). BK virus was first recovered in 1971 from a patient with a renal transplant. This new virus, a member of the polyoma virus subgroup of papovaviruses, was designated BK (the patient's initials). BK virus is ubiquitous, and seroprevalence in childhood ranges from 60% to 100%. The kidney is thought to be the reservoir for BK virus and transmission may be fecal-oral.³ BK virus is an important pathogen in immunocompromised patients. In solid organ and bone marrow transplant patients, BK virus can cause nephritis (and loss of a transplanted kidney) and/or hemorrhagic cystitis (and possibly bladder tamponade). There is little information on treating BK virus infections with antiviral agents such as cidofovir, and the usual therapeutic approach is a reduction in immunosuppression.⁴

The majority of children become seropositive for BK virus during the first decade of life. The initial BK virus infection can occur without signs or symptoms; however, BK virus infection can also present as a urinary tract infection. There are only 3 case reports of BK virus infection in nonimmunosuppressed hosts. The first case⁵ was a 5-year-old boy who presented with dysuria, frequency, and terminal hematuria. When the bacterial culture was negative, urine cytology was performed and showed epithelial cells with intranuclear inclusions. Electron microscopy revealed papovavirus-like particles. Symptoms resolved in a week. The second case⁶ was a 3-year-old boy with nonhemorrhagic cystitis whose urine cytology showed nuclear changes typical of polyomavirus. Viral culture (using human fetal glial cells) showed vacuolization typical of BK virus. Also, electron microscopy and serologies were consistent with a BK viral infection. The child made an uneventful recovery. The third case⁷ was a 5-year-old boy with frequency, hematuria, and negative bacterial urine culture. Urine cytology was abnormal and PCR was positive for BK virus. This third patient was asymptomatic in 2 weeks.

BK viral infection can be devastating in a transplant patient. In nonimmunosuppressed children, BK virus infection can cause dysuria and hematuria. BK urinary tract infection can be confirmed using urine cytology and immunochemical staining, PCR, electron microscopy, or viral culture. BK virus cystitis has now been documented in 4 previously normal children, and in all cases it was benign and resolved spontaneously within 2 weeks.

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