Citation Information

Journal: Pediatric Infectious Disease Journal

Article: SEVERE PERTUSSIS IN INFANTS: ESTIMATED IMPACT OF FIRST VACCINE

Author: Foxwell

ISSN: 08913668

Volume: 30

Issue: 2

Year: 2011

Pages: 161 - 163

Citation Source: null

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SEVERE PERTUSSIS IN INFANTS

ESTIMATED IMPACT OF FIRST VACCINE DOSE AT 6 VERSUS 8 WEEKS IN AUSTRALIA

Alice Ruth Foxwell, MSc, PhD,*† Peter McIntyre, MB BS, PhD;‡ Helen Quinn, MAE, PhD,‡ Katrina Roper, MAE, PhD,* and Mark S. Clements, PhD‡

Abstract: We estimated the potential benefits of advancing the first dose of pertussis vaccine for infants from 8 to 6 weeks of age, using Australian national disease databases. Infants had notification rates 3-fold greater than the general population and accounted for 52% of recorded hospitalizations. Infants 1 and 2 months of age had notification rates 3.5 times (95% CI 2.7–4.5) higher than infants 3 to 11 months of age. Estimation of acceleration of the vaccine to 6 weeks of age reduced average notification, hospitalizations, and hospital bed-days by 8%, 9%, and 12%, respectively, with larger reductions in an epidemic year.

Key Words: vaccine, pertussis, whooping cough, immunization

Accepted for publication July 27, 2010.

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Supported by the Australian Government Department of Health and Ageing (to A.R.F., Master of Applied Epidemiology program). The National Centre for Immunisation Research and Surveillance for Vaccine Preventable Diseases is supported by the Australian Government Department of Health and Ageing, the New South Wales Department of Health and the Children’s Hospital at Westmead.

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DOI: 10.1097/INF.0b013e3181f43096

METHODS

Pertussis notifications with diagnosis date between January 1 1999 and December 31 2008 were extracted from the Australian National Notifiable Diseases Surveillance System. Hospitalizations coded in any field as whooping cough with an admission date between January 1 1997 and December 31 2006 were extracted from the Australian Institute of Health and Welfare Hospital Morbidity Database. Deaths were derived from notifications so coded in the National Notifiable Diseases Surveillance System.

Incidence rates were calculated using Australian Bureau of Statistics midyear population estimates in the denominator, with the assumption that the population for each month of age was one-twelfth that of the total population less than 1 year of age. Bed-days were calculated by summing total days in hospital for each age group.

Negative binomial regression was used to compare incidence rates across age groups using rate ratios. χ² was used for trend comparison. Ninety-five percent confidence intervals (CI) and P values were calculated using Intercooled STATA version 9. P values less than 0.05 were considered statistically significant.

Following methodology established by Shinall et al, the effect of vaccine acceleration was estimated by using the average rate per month of pertussis notification or hospitalization multiplied by the number of live births in 2007 in Australia (285,213) to obtain the expected number of pertussis notifications or hospitalizations by month of age. Benefits were calculated using an incidence shift in whooping cough rates, hospital admissions, or bed-days of 2 weeks from the second to the fourth month. Benefits accrue because of the decrease in notifications and hospitalizations reported each month from 1 month to 3 months of age. Estimations assumed an equivalent immune response to DTPa vaccine at 6 and 8 weeks of age and that the timeliness of vaccine coverage under a 6- and 8-week first dose recommendation would be similar. The difference in rates of notifications and hospitalizations equated the current rate minus the estimated rate. Similar calculations were applied for estimating bed-days saved. The primary series for pertussis containing vaccine in Australia is given at 2, 4, and 6 months of age, although 1 jurisdiction (New South Wales) has recommended commencing at 6 weeks since 2009.

RESULTS

Pertussis Notification and Hospitalization in Infants Less Than 1 Year Old. For the period 1999 to 2008, the notification rate for pertussis in children less than 1 year of age was 2.6 times that of the general population. In the 10 years, between 1997 and 2006, infants aged less than 1 year represented 52.4% (3052 cases) of all recorded whooping cough hospital admissions. All the notifications and 41% (1228) of hospital admissions had Bordetella pertussis attributed as the causative agent.

When notification rates were examined by 1 month age brackets, those aged 1 and 2 months were 3.5 times more likely to be notified for pertussis than those aged 3 to less than 12 months (Table 1). Compared with infants aged between 3 and less than 12 months, the incidence rate ratio was 3.5 (95% CI 2.7–4.6) for those aged 1 month, 3.4 (95% CI 2.6–4.5) for those aged 2 months, and 2.0 (95% CI 1.5–2.7) for those aged 3 months (P = 0.063) and 49% (P = 0.004) less likely to contract pertussis than those at 2 months. A similar pattern was seen for hospital admissions (34% and 57% reduction) and overall hospital bed-days (50% and 73% reduction) (Table 1). If notifications and total hospital admissions between 1999 and 2006 were compared, a larger proportion of infants aged 1, 2, and 3 months were hospitalized than other infants less than 1 year. Among hospitalized infants, there was a

<table>
<thead>
<tr>
<th>Year</th>
<th>0 Months</th>
<th>1 Month</th>
<th>2 Months</th>
<th>3 Months</th>
<th>4 Months</th>
<th>5 Months</th>
<th>6–11 Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean notification rate per 100,000 (range)</td>
<td>133 (52, 238)</td>
<td>245 (91, 489)</td>
<td>232 (110, 447)</td>
<td>150 (22, 287)</td>
<td>116 (43, 299)</td>
<td>68 (22, 155)</td>
<td>50 (15, 94)</td>
</tr>
<tr>
<td>Mean hospitalization rate per 100,000 (range)</td>
<td>128 (62, 264)</td>
<td>386 (169, 810)</td>
<td>339 (164, 610)</td>
<td>224 (84, 602)</td>
<td>146 (48, 297)</td>
<td>73 (31, 185)</td>
<td>27 (15, 73)</td>
</tr>
<tr>
<td>Mean LOS (95% CI) (d)</td>
<td>6.4 (8.10, 9.7)</td>
<td>8.8 (6.3, 7.4)</td>
<td>6.4 (14.7, 8.3)</td>
<td>4.8 (4.3, 4.6)</td>
<td>4.1 (3.6, 4.6)</td>
<td>3.5 (2.9, 4.6)</td>
<td>4.7 (3.5, 6.6)</td>
</tr>
<tr>
<td>Total Bed-days per year (95% CI) (d)</td>
<td>1.25 (1.10, 1.30)</td>
<td>5.30 (4.87, 5.72)</td>
<td>4.50 (3.96, 5.25)</td>
<td>2.28 (2.03, 2.53)</td>
<td>1.27 (1.11, 1.42)</td>
<td>0.53 (0.43, 0.61)</td>
<td>0.27 (0.16, 0.38)</td>
</tr>
<tr>
<td>More than 5 d in hospital (%)</td>
<td>52</td>
<td>45</td>
<td>37</td>
<td>28</td>
<td>24</td>
<td>19</td>
<td>22</td>
</tr>
</tbody>
</table>

CI indicates confidence interval.

clear trend for decreasing length of hospital stay with increasing age. The proportion of infants spending more than 5 days in hospital declined from 52% for those less than 4 weeks of age to 23% at 6 months (P < 0.001, Table 1) and for more than 10 days in hospital from 29% to 5%, respectively.

Effect of Vaccine Acceleration. It was estimated that accelerating the first dose of the DTPa vaccine from 2 months to 6 weeks of age would result in an annual reduction of 11 notifications (8%), 17 hospitalizations (9%), and 151 bed-days (12%). The number needed to vaccinate to achieve this reduction would be 25,465 for notifications and 16,679 for hospitalizations.

Whooping cough occurs in epidemic cycles, with a substantially greater burden and potential for public concern in an epidemic year. If calculated for 2001, an epidemic year common to both data sets, we find a greater reduction than the mean with 12% in notifications, 10% in hospitalizations and 12% in hospital bed-days.

Of the 11 deaths identified in the study period, 4 (36%) would have had the opportunity to be immunized at least 10 days before date of diagnosis of disease with 1 dose of vaccine if the first dose were to be advanced to 6 weeks of age.

DISCUSSION

Infants in their first 3 months of life are at significantly greater risk than any other age group of acquiring severe pertussis, as measured by hospitalization, length of hospital stay, and death. Potential measures to reduce the burden of early infant pertussis may be both indirect, such as maximizing the timeliness and population coverage of infant vaccination and vaccination of adults in contact with infants, including maternal vaccination, and direct such as earlier infant vaccination.

Our findings are in keeping with a recent analysis using aggregated US data which estimated that accelerating the first vaccine dose by 2 weeks reduced notified whooping cough cases by 8% and hospitalizations by 9%. Additionally, the longer hospital stay seen among the youngest infants could be reduced by 12% of hospital bed-days per year. We also estimated the potential benefits of an earlier first vaccine dose in epidemic years and found that the additional estimated benefit increased by 50% (from 8% to 12%) for notifications and by 11% (from 9% to 10%) for hospitalizations compared with an interepidemic period. As with the US study, the reduction in cases occurs mainly between the second and fourth month of life and depends on the assumption that some degree of protection against severe pertussis accrues after even 1 dose. A limitation of the databases used is that there is potential for under-ascertainment (if the most sensitive diagnostic test was not used) and for misclassification (if some cases coded as pertussis on clinical grounds without laboratory confirmation were hospitalized for other reasons). Furthermore, the estimated reduction in hospitalizations may be less than 12% as only 41% of hospital admissions were coded as being specifically due to B. pertussis.

The reductions between month 2 and 4 and then 4 and 6 in notifications (49% and 59%, respectively) and hospitalizations (57% and 82%, respectively) provide some evidence for the efficacy of the first and the second dose of a pertussis containing vaccine. This trend was also noted in the analysis of hospitalization rates by Cortese et al and in notification rate data by Shinnall et al. Uncertainty regarding immune response is greater for acceleration of the third dose of vaccine, than for the first dose for postimmunization levels of pertussis, diphtheria, and tetanus antibodies. With respect to the first dose of vaccines including acellular pertussis antigens, administration from 6 weeks of age is approved by regulatory authorities in North America, Europe, New Zealand, and Australia, although little direct comparative data between first doses at 6 or 8 weeks are available. With respect to subsequent doses, some European countries have accelerated primary schedules with the third dose at 4 months of age, while others do not give the third dose, as a booster, until 12 months of age.

There is evidence of lesser responses to both pertussis antigens and other antigens, particularly the protein conjugate polysaccharide vaccines, when the third dose is given at 4 months. In the light of these concerns, and the evidence of good protection against pertussis after 2 doses (when given at 2 and 4 months of age),10,11 should the first dose be accelerated to 6 weeks, caution would be required regarding a third dose any earlier than 6 months of age. With respect to the implementation of an earlier first dose, it is likely that if parents and vaccine providers were convinced about the benefits, this would be straightforward. This is because most industrialized countries have existing recommendations for a routine postnatal visit at approximately 6 weeks, which could be integrated with commencement of the infant vaccine schedule.

ACKNOWLEDGMENT

The authors thank the State and Territory Health Authorities and the Office of Health Protection for provision of data from the National Notifiable Diseases Database and the Australian Institute of Health and Welfare for provision of data from the Morbidity Database.

REFERENCES

CASES

Clinical and laboratory features of the 5 cases are presented in Tables 1 and 2. Each of these patients eventually was hospitalized for evaluation of fever. All of our cases presented after at least 1 week of fever following multiple evaluations and treatments for potential alternative diagnoses. Of the 5 patients, 4 had rashes. Headache, abdominal pain, myalgias, and emesis were prominent symptoms. All had elevated C-reactive protein, mild hepatitis, and mild hyponatremia. Cases 1 and 2 presented with the more classic picture of prolonged fever, rash, and headache in a teenager child. Both responded rapidly to empiric doxycycline therapy, becoming afebrile within 24 hours. Case 3 had mild temperature typically seen in the younger child. Case 4 demonstrates the diagnostic difficulty of considering the diagnosis when rash is not present. This child was admitted to the pediatric intensive care unit because of severe thrombocytopenia and concern for possible hemolytic-uremic syndrome. Case 5 highlights the clinical overlap between murine typhus and Kawasaki disease as this patient met full criteria for Kawasaki disease, including an isolated cervical lymphadenopathy, and received intravenous immunoglobulin and aspirin before the diagnosis of murine typhus was performed. All patients had diagnosis of murine typhus confirmed by indirect fluorescent antibody testing (IFA), with acute tiers sent to commercial laboratories (Specialty Laboratories in Valencia, CA for cases 1, 3, and 4) and convalescent tiers to the Orange County Health Department.

DISCUSSION

Endemic typhus, first described in 1926, is a zoonotic disease with a rat reservoir and a flea vector, most commonly Rhipicephalus sanguineus. Humans are incidental hosts. Historically, it has been considered an urban disease caused by overcrowding and poor sanitation. Currently less than 100 cases are reported annually in the United States. However, the exact prevalence of murine typhus is difficult to determine because of under reporting and under-diagnosis. Aside from a 2002 outbreak in Hawaii, cases are mostly limited to Texas and Southern California. In 2009 in California, there were 25 reported cases of typhus, 6 of which were in Orange County. There is evidence that typhus exists in other regions, as demonstrated by Marshall's data of seropositive children in Oklahoma, Kentucky, and Kansas. As early as 1970, researchers noticed a trend in typhus cases in suburban locations rather than the typical urban setting. Adams et al. documented an increase in R. typhi seropositive animals, specifically opossums, in the Los Angeles suburbs. Opossums carry a different species of flea, Ceratophyllus felis or “cat flea,” which is ubiquitous and infects peridomestic animals such as opossums, skunks, and free ranging cats and dogs. It has been shown that C. felis can transmit R. typhi to humans. Since suburban dwellers are more likely to have contact with peridomestic animals, murine typhus is now being maintained in the suburban areas of Southern California and Texas. There is also increasing evidence that many of the clinically diagnosed cases of murine typhus may, in fact, be caused by Rickettsia felis, a recently discovered pathogen.

Symptoms of endemic typhus may be mild, particularly in younger children, and seldom last for more than 2 weeks, leading to under diagnosis. However, visceral involvement, such as aseptic meningitis, or endocarditis, can occur. During a recent outbreak in Austin, Texas of 53 patients, only 8 of whom were children, 70% were hospitalized, with 30% in the intensive care unit. Untreated severe disease can be fatal in 4% of cases.

Our cases exhibited features consistent with those previously described in the literature. The most common symptoms in

A CLUSTER OF PEDIATRIC ENDEMIC TYPHUS CASES IN ORANGE COUNTY, CALIFORNIA

Jennifer S. Green, MD,∗ Jasjit Singh, MD,† Michele Chang, MD,‡ Felice C. Adler-Shohet, MD,† and Nayar Ashouri, MD∗

Abstract: Murine typhus is a mild febrile illness caused by Rickettsia typhi, generally confined to Texas and Southern California. Clinicians should consider early treatment with doxycycline when presented with a child having protracted fever, rash, and headache. We present 5 pediatric cases and a literature review highlighting the changing epidemiology and diagnostic difficulty of typhus.

Key Words: murine typhus, endemic typhus, Rickettsia typhi, pediatric Accepted for publication July 27, 2010.

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DOI: 10.1097/INF.0b013e3181f4ce25

Murine, or endemic typhus, is a febrile illness caused by Rickettsia typhi, an obligate intracellular Gram-negative organism. Fever may be accompanied by severe headache and myalgias. A discrete maculopapular rash typically appears on days 5 to 7 of illness. Since symptoms have considerable overlap with a variety of illnesses, particularly viral syndromes, the diagnosis is often not considered. We present 5 pediatric cases of confirmed murine typhus in Orange County, California, which highlight the need to consider the diagnosis of endemic typhus in the child with prolonged fever and rash. These children received extensive and costly evaluations before the diagnosis of typhus was considered. The changing epidemiology and review of the literature are intended to aid the clinician in considering this infection in the diagnosis of the child with fever of unknown origin.

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### TABLE 1. Clinical Features

<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Duration of Symptoms at Presentation</th>
<th>Fever (Tmax)</th>
<th>Rash</th>
<th>Headache</th>
<th>Abdominal Pain</th>
<th>Hepatomegaly</th>
<th>Other Symptoms</th>
<th>Exposures</th>
<th>Presumed Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15 yr</td>
<td>9 d</td>
<td>Yes (39.4°)</td>
<td>Yes</td>
<td>Yes (frontal)</td>
<td>Yes (severe)</td>
<td>Yes</td>
<td>Myalgias, emesis, diarrhea</td>
<td>Cats at home, no flea bites</td>
<td>EBV, constipation, mycoplasma, viral hepatitis, adenovirus</td>
</tr>
<tr>
<td>2</td>
<td>14 yr</td>
<td>10 d</td>
<td>Yes (39.4°)</td>
<td>Yes</td>
<td>Yes (diffuse)</td>
<td>Yes</td>
<td>No</td>
<td>Myalgias, emesis</td>
<td>Cats, brother with fever and headache but no rash</td>
<td>Pneumonia, EBV, CSF, Kawasaki</td>
</tr>
<tr>
<td>3</td>
<td>3.5 yr</td>
<td>9 d</td>
<td>Yes (40.5°)</td>
<td>Yes</td>
<td>Yes (diffuse)</td>
<td>No</td>
<td>Yes</td>
<td>Conjunctivitis, chapped lips</td>
<td>Kitten, recent scratch to patient</td>
<td>EBV, WNV, mycoplasma</td>
</tr>
<tr>
<td>4</td>
<td>14 yr</td>
<td>10 d</td>
<td>Yes (40.2°)</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Myalgias, emesis, diarrhea, cough</td>
<td>Cats, no flea bites</td>
<td>EBV, CSF, toxoplasma</td>
</tr>
<tr>
<td>5</td>
<td>5 yr</td>
<td>7 d</td>
<td>Yes (40°)</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Arthralgias, conjunctivitis, chapped lips, decreased energy, emesis, isolated lymphadenopathy</td>
<td>Cats, no flea bites</td>
<td>Pharyngitis, Kawasaki</td>
</tr>
</tbody>
</table>

CSD indicates Cuts-Scratch disease; EBV, Epstein-Barr virus; WNV, West Nile virus.

### TABLE 2. Laboratory Features

<table>
<thead>
<tr>
<th>Case</th>
<th>WBC (Cell/µL)</th>
<th>CRP (mg/dL)</th>
<th>ESR (mm/h)</th>
<th>AST/ALT (µ/L)</th>
<th>Sodium (mMol/L)</th>
<th>Acute <em>R. typhi</em> Titters (IgM/IgG)</th>
<th>Convalescent <em>R. typhi</em> Titters (IgM/IgG)</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1</td>
<td>11,200 (69% neutrophil, 5% band)</td>
<td>11.84</td>
<td>16</td>
<td>45/60</td>
<td>134</td>
<td>1:1024/1:1024</td>
<td>1:5120/1:36384</td>
<td>Doxycycline on day 11 of illness</td>
</tr>
<tr>
<td>Case 2</td>
<td>6000 (65% neutrophil, 0% band)</td>
<td>13.8</td>
<td>Not done</td>
<td>68/60</td>
<td>125</td>
<td>1:64/1:256</td>
<td>1:5120/1:36384</td>
<td>Doxycycline on day 10 of illness</td>
</tr>
<tr>
<td>Case 3</td>
<td>6000 (48% neutrophil, 26% band)</td>
<td>7.56</td>
<td>23</td>
<td>66/40</td>
<td>130</td>
<td>1:256/1:256</td>
<td>1:1024/1:1024</td>
<td>No treatment</td>
</tr>
<tr>
<td>Case 4</td>
<td>13,890</td>
<td>14.43</td>
<td>38</td>
<td>112/66</td>
<td>125</td>
<td>1:64/1:3054</td>
<td>1:64/1:1256</td>
<td>No treatment</td>
</tr>
<tr>
<td>Case 5</td>
<td>11,800 (69% neutrophil, 16% band)</td>
<td>6.06</td>
<td>12</td>
<td>75/654</td>
<td>133</td>
<td>1:256/1:256</td>
<td>Inaccurate due to IVIG</td>
<td>IVIG and aspirin, then doxycycline on day 10 of illness</td>
</tr>
</tbody>
</table>

WBC indicates white blood cells; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; AST, aspartate aminotransferase; ALT, alanine aminotransferase; *R. typhi*, *Rickettsia typhi*; IgM, immunoglobulin M; IgG, immunoglobulin G; IVIG, intravenous immunoglobulin.
a case series of 30 pediatric patients were fever, rash, and headache, and the most common laboratory abnormalities were elevated erythrocyte sedimentation rate, elevated transaminase values, hyperammonemia, and leukopenia with a left shift.9 Our patients had similar findings and, additionally, abdominal pain was common. Unfortunately, patients without these common symptoms may not be recognized such as was case 4 who lacked a rash. Lymphadenopathy, especially isolated lymphadenopathy, is not a common finding,9 thus adding to the complexity of case 5. The difficulty comes in confirming the clinical suspicion of typhus, as these symptoms and laboratory values are nonspecific and frequently lead to extensive infectious workup and treatments before to serologic confirmation. Because typhus serologies are performed only in certain specialty laboratories, results are often delayed making empiric administration of doxycycline imperative if the diagnosis is suspected to prevent severe or life-threatening disease.10 Doxycycline is considered a safe and superior therapy in children less than 8 years old, regardless of risk of tooth staining.11 IFA and enzyme immunoassays have largely replaced the Weil Felix agglutination test and peak at about 4 weeks after infection. A 4-fold titr increase between acute and convalescent serum specimens is diagnostic. IFA, the standard diagnostic test, identifies antibodies to the Rickettsia heat-labile protein antigens and lipopolysaccharide antigens.12 Enzyme immunoassay uses antigen-coated wells to identify different types of Rickettsial infections. The different Rickettsial species are so closely related that there is significant cross-reactivity, especially between R. typhi and R. felis, which are 98.5% homologous.12 A polymerase chain reaction test that amplifies the spotted fever group genetic segment or the typhus group segment during the acute phase of infection is not commercially available.1

CONCLUSION
Clinical manifestations of endemic typhus can mimic other protracted febrile illnesses such as viral infections and noninfectious processes such as Kawasaki disease. Clinicians should consider the diagnosis even in patients without rash, who have consistent clinical and laboratory features, particularly those residing in endemic areas like southern California, southern Texas, and Hawaii. Empiric doxycycline while awaiting confirmatory serologic results can lead to rapid improvement and avoid life-threatening complications.

REFERENCES

NEUROLOGIC MANIFESTATIONS OF PEDIATRIC NOVEL H1N1 INFLUENZA INFECTION

Neil Reillosa, MD,* Karen C. Bloch, MD, MPH,† Andi L. Shane, MSc, MD, MPH,*‡ and Roberta L. DeBiasi, MD,*§

Abstract: Reported neurologic manifestations of novel H1N1 influenza have included seizure, meningoencephalitis, and acute necrotizing encephalopathy. We describe the first series of pediatric patients presenting during the second wave of the US novel H1N1 pandemic, with reported seizures, severe encephalopathy/encephalitis, and acute disseminated encephalomyelitis. In addition to prominent radiographic abnormalities, we provide the first observation and description of associated cerebrospinal fluid abnormalities.

Key Words: novel H1N1 influenza, encephalitis, central nervous system

Accepted for publication July 20, 2010.

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DOI: 10.1097/INF.0b013e3181f2def6

Neurologic complications occurring in association with seasonal influenza type A (H1N1 and H3N2) and influenza type B viruses have been well documented in children. A broad spectrum of manifestations have been described, including seizures, encephalitis, acute disseminated encephalomyelitis, Guillain-Barré syndrome, transverse myelitis, encephalopathy, and acute necrotizing encephalopathy (ANEn).1-4

The first report of neurologic disease in association with novel H1N1 influenza A (H1N1) infection occurred during the first pandemic wave (May 2009), in 4 pediatric patients with symptoms ranging from seizures to mild encephalopathy. However, cerebrospinal fluid (CSF) pleocytosis was absent in these patients, radiologic evidence of brain parenchymal involvement was absent in 3 of the 4 patients (with only nonspecific white matter changes noted in 1 of the 4), and all patients recovered fully.5 Subsequently, 5 additional cases have been reported; an infant with seizure,6 an adolescent with meningoencephalitis,7 and 3 children with ANEn.8-10 with a fatal outcome in a 7-year-old previously healthy infant of Asian descent with ANEn.8 Radiologic abnormalities were described in several of these cases,8-10 but CSF pleocytosis was absent, as in the previously described reports.1-5

The present report includes a series of pediatric patients presenting during the second wave of the US H1N1 pandemic, with a more severe spectrum of neurologic manifestations includ-

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ing protracted seizures, severe encephalopathy/encephalitis, and acute disseminated encephalomyelitis (ADEM), all of which occurred in previously normal children. In addition to prominent radiographic abnormalities, these cases were unique based on concomitant CSF abnormalities in 2 of 3 cases.

METHODS

Case Identification. We retrospectively identified 3 pediatric patients with confirmed H1N1 infection and associated neurologic complications hospitalized during the second wave of the US pandemic (August-November 2009). All patients had preceding or concurrent influenza-like illness (ILI) associated with seizures, encephalopathy, or encephalitis in conjunction with a confirmatory laboratory test result for influenza A, without an alternative etiology identified. CSF polymerase chain reaction (PCR) was performed for herpes simplex virus (HSV) and enterovirus (EV) on all patients and for novel H1N1 influenza in 2 patients using the proFlu-M multiplex real-time PCR (RT-PCR) assay (Prodesse, Madison, WI), according to the manufacturers’ instructions. Two of the 3 patients (A and C) were enrolled in the Tennessee Unexplained Encephalitis Study and had extensive additional testing of CSF and serum, with no alternative infectious etiology identified.

RESULTS

Case Descriptions.

Patient A. On August 27, 2009, a previously healthy, sickle cell trait-positive, 5-year-old black boy was admitted with a 1-day history of confusion and irritability, preceded by a 5-day history of upper respiratory symptoms including an oral temperature of 39.4°C, cough, rhinorrhea, headache, and myalgias. He had positive rapid influenza A testing at the referring urgent care facility, subsequently confirmed on admission by nasopharyngeal viral antigen panel and nasal-swab RT-PCR. He had not received seasonal or novel H1N1 vaccines in 2009. CSF analysis revealed pleocytosis, elevated protein, and normal glucose values. CSF RT-PCR for influenza A was negative (Table 1). His initial physical examination was unremarkable, but his mental status declined, progressing to unresponsiveness to verbal commands, grimacing, twitching, and myoclonus. Electroencephalogram (EEG) revealed complex partial status epilepticus and he was treated with antiepileptic medications (AEM). He required transfer to the intensive care unit, was intubated and maintained in a pentothal-induced coma from hospitalization day (HD) 7. Initial head computed tomography (CT) was unremarkable. However, brain magnetic resonance imaging (MRI) on HD 8 revealed subcortical white matter T2 hyperintensities with minimal enhancement of the right insular cortex and parietal cortex, consistent with ADEM. He received a full course of oseltamivir (HD 1–5), was transitioned to ribavirin on HD 13, and extubated on HD 19. After clinical improvement, he was discharged to a rehabilitation facility on HD 27. To date, he has mild residual speech deficits that were not present premorbidly.

Patient B. On October 19, 2009, a previously healthy 23-month-old Hispanic male was admitted with 6 episodes of presumptive seizure beginning at his home and continuing in the emergency department, characterized by generalized body stiffening with tonic-clonic jerks of his extremities and associated eye-rolling, lasting approximately for 2 to 3 minutes. Nine days before admission, he was treated with oral antibiotic therapy for a presumed left ear infection with associated subjective fever, cough, and rhinorrhea. He had not received H1N1 vaccine. On physical examination, he was febrile to 39.3°C with an unremarkable neurologic examination except for 4 to 5 beats of clonus at the ankle bilaterally. Respiratory viral antigen panel testing and RT-PCR of the upper respiratory tract were negative.

Table 1. Summary of Clinical, Radiographic, and Laboratory Features

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Summary of Clinical, Radiographic, and Laboratory Features</th>
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<tbody>
<tr>
<td><strong>Patient A</strong></td>
<td><strong>5-year-old boy</strong></td>
</tr>
<tr>
<td><strong>Constitution</strong></td>
<td>Confusion, irritability, diminished mentation, myoclonus, seizures</td>
</tr>
<tr>
<td><strong>Time to Admission</strong></td>
<td>95 minutes</td>
</tr>
<tr>
<td><strong>CSF Profile</strong></td>
<td>WBC: 5 cells/µL, RBC: 5 cells/µL, glucose: 45 mg/dL, protein: 20 mg/dL, no mimic</td>
</tr>
<tr>
<td><strong>MRI</strong></td>
<td>Bilateral signal &amp; heterogeneity in the brainstem and periventricular white matter</td>
</tr>
<tr>
<td><strong>Brain-Add FLAIR</strong></td>
<td>Enhanced lesion in the periventricular white matter</td>
</tr>
</tbody>
</table>

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nasopharyngeal specimens were both positive for influenza A. CSF analysis was unremarkable, and CSF PCR testing for HSV and EV was negative (Table 1). After being admitted, the patient continued to have seizures, in absence of fever, and was treated with AEM. EEG revealed bilateral frontal spike wave signaling with bilateral frontal interictal spikes. Initial noncontrast head CT showed no parenchymal abnormalities, although opacification of bilateral mastoid cells without bony erosion was observed. Brain MRI was not performed, as he improved by HD4 and remained seizure-free on AEM. He also received a full course of oseletamivir (HD 1-5). He was discharged to home on HD5. Follow-up examination and outpatient brain MRI 2 months after discharge were normal.

**Patient C.** On October 23, 2009, a previously healthy 9-year-old black boy was admitted with a 3-day history of progressive altered mental status. Approximately 9 days before admission, he had subjective fevers, headache, vomiting, sore throat, mild cough, and congestion. He was evaluated at an urgent care center, along with his aunt and cousin, all of whom had ILI and positive rapid testing for influenza. He was not treated with antiviral therapy, but did consume one dose of oseletamivir which he had prescribed for his aunt. He had not received the H1N1 vaccine. Beginning 3 to 4 days before admission, he developed gradually progressive fatigue/sleepiness and waxing/waning level of responsiveness. He developed abnormal, nontensive, nonsensical speech, and eventually became mute. On the day of admission, his family noted "crossed eye" gaze, and his eyes rolling to the back of his head. PCR testing of his nasopharyngeal specimen was negative. CSF analysis revealed pleocytosis with a lymphoic predominance, elevated protein, and normal glucose values (Table 1). The CSF PCR for HSV, EV, and influenza A was negative. An EEG showed diffuse synchronous and asynchronous background slowing with intermittent rhythmic delta waves consistent with moderate diffuse encephalopathy. An initial noncontrast CT of his brain was within normal limits. However, a brain MRI performed on HD 2 revealed bilateral increased signal and heterogeneity of the basal ganglia (including the head of the caudate and putamen), the thalamus and splenium of the corpus callosum on T2 FLAIR sequences, without gadolinium enhancement. He gradually improved and made a complete recovery without any neurologic sequelae by the time of hospital discharge on HD 6. He completed a 5-day course of oseletamivir therapy.

**DISCUSSION**

Our series is the first to describe the neurologic manifestations of nH1N1 infection during the second wave of the US pandemic, and the first to observe and describe associated CSF abnormalities, which have not been noted in prior reports. The nH1N1 pandemic, in contrast to seasonal influenza epidemics, disproportionately affected a younger population, and presents an important opportunity to observe the neurologic manifestations of this newly emerged virus. In the first wave of the US pandemic, 4 children ranging from 7 to 17 years of age with mild to moderately severe neurologic abnormalities were reported, all of whom made a full recovery.2 No CSF abnormalities were observed in these patients, and 3 of the 4 had normal neuroimaging studies. Five additional single cases have subsequently been published that have broadened observations to include a younger age group, significant radiologic abnormalities, and in 1 case, death, although CSF pleocytosis was absent in all of these cases.6-10

Our series of cases illustrates several features that have not been previously appreciated in association with nH1N1 neurologic disease:

1. A predominantly lymphocytic/monocytic CSF pleocytosis was observed in 2 of the 3 patients, without detection of influenza virus by PCR. Two patients had an elevated CSF protein, and all had glucose CSF values that were within normal range.
2. The time frame within which our series of patients presented with neurologic symptoms related to their respiratory symptoms was quite variable and extends the period after ILI within which nH1N1-infected children may present with CNS manifestations. In 1 case, onset of severe encephalopathy was delayed until after full resolution of ILI symptoms, peaking 9 days after onset. This is in contrast to prior reports, in which neurologic symptoms generally occurred concomitant with respiratory symptomology.
3. The pattern of radiographic findings in Patient C is consistent with recent reports,1,6 and include marked bilaterally symmetric basal ganglia and deep nuclei abnormalities. However, Patient A illustrates for the first time that that widespread white matter abnormalities, typical of ADEM, may also occur in association with nH1N1 infection.
4. Despite the severity of symptomology observed in our cases, a favorable outcome occurred. In contrast, the outcomes of patients presenting with seasonal influenza-associated neurologic complications have been highly variable, ranging from complete recovery without sequelae to death, particularly in Asian patients with ANE, in which up to 30% mortality has been observed.4

In summary, this series broadens our understanding of the scope of neurologic manifestations and laboratory abnormalities associated with pediatric nH1N1 infection. In concert with other reported cases, lack of detection of viral genome in the CSF at the time of symptom onset may be important in differentiating immune-mediated or postinfectious mechanisms of CNS injury rather than direct infection. Continued vigilance is warranted, especially for late-onset neurologic manifestations, as this newly emerged virus adapts to the human population.

**REFERENCES**

PNEUMOCOCCAL MENINGITIS IN FRENCH CHILDREN BEFORE AND AFTER THE INTRODUCTION OF PNEUMOCOCCAL CONJUGATE VACCINE

Corinne Levy, MD, Emmanuelle Varon, MD, PhD, Edouard Bingen, PhD, Aurélie Lécuyer, MD, Michel Boucharat, MD, Robert Cohen, MD, and The Bacterial Meningitis Study Group

Abstract: In France, despite a high rate of pneumococcal conjugate vaccine coverage, the number of cases of pneumococcal meningitis in children did not decline significantly between 2001-2002 (n = 264) and 2007-2008 (n = 244). A decline was observed among children <2 years old (185 [70.1%] to 134 [54.9%]; P = 0.0004), but was countered-balanced by an increase among children ≥2 years old (79 [29.9%] to 110 [45.1%]; P = 0.0004). Mean age increased significantly, from 2.3 (median 0.8) to 3.8 (median 1.5) years. After pneumococcal conjugate vaccine 7 implementation, a wide diversity of serotypes implicated in pneumococcal meningitis was observed; serotypes 19A and 7F were the most frequent.

Key Words: pneumococcal meningitis, children

Accepted for publication July 30, 2010.

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The authors have no conflicts of interest to declare.

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Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal’s Web site (www.pidj.com).

DOI: 10.1097/INF.0b013e3188f4e969

In France, Streptococcus pneumoniae is the leading cause of bacterial meningitis in children aged less than 1 year and the second cause (after Neisseria meningitidis) in older children. Many countries have adopted a 7-valent pneumococcal conjugate vaccine (PCV7) as part of their routine vaccination programs, and several studies have described its impact on invasive pneumococcal disease (IPD). Since PCV7 was introduced in the United States, the incidence of IPD has fallen, not only in the vaccinated target population but also among persons too young or too old to receive the vaccine. In contrast, few studies have examined the impact of PCV7 on pneumococcal meningitis. In the United States, between 1998–1999 and 2006–2007, following PCV7 implementation for children aged <5 years, a larger decline was observed in bacteremia without focal infection (85%) than in pneumococcal meningitis (64%). In France, PCV7 was introduced for children younger than 2 years of age in March 2002 (immunization schedule 3 + 1), and started to be reimbursed by the national health insurance in January 2003. The PCV7 vaccination coverage has increased slowly: at least 1 dose of PCV7 had been received by 56% of children less than 1 year old in 2004, compared with 64% in 2006, 73% in 2007, and 86% in 2008. This gradual PCV7 uptake was probably related to the complexity of the initial guidelines, which recommended vaccination only for children less than 2 years of age who were considered at risk because of underlying health disorders or living conditions (children in day care with at least 2 other children for more than 4 hours per week, children in families with more than 2 children, and children breast-fed for less than 2 months). In 2006, vaccination was extended to all children less than 2 years of age.

Eight years ago the Pediatric Infectious Diseases Group of the French Pediatrics Society set up an active surveillance network to monitor the clinical and biologic features of bacterial meningitis. Here we analyze the impact of PCV7 on pneumococcal meningitis in France, based on data from this nationwide survey.

METHODS

From January 2001 to December 2008, 252 pediatric wards (68% of cities with a pediatric ward in France) working with 168 microbiology departments throughout France were asked to report all cases of bacterial meningitis. Three times a year, a clinical investigator in each participating ward was contacted for information on new cases, or to confirm the lack of such cases. After patient discharge, a standardized data form was completed by a designated clinical investigator and sent to the investigating center. The following diagnostic criteria were used for S. pneumoniae meningitis in children aged from 1 day to 15 years: clinical signs associated with positive cerebrospinal fluid (CSF) culture and/or positive CSF antigen testing, and/or positive CSF polymerase chain reaction (PCR), and/or culture positivity of a normally sterile body site associated with CSF pleocytosis.

The following data were collected: CSF analysis, blood culture, date of birth, gender, vaccine status, underlying conditions (eg, congenital or acquired meningeval breach, valve, recurrent meningitis, cochlear implantation, asplenia or sickle cell disease or spleenectomy, immunodeficiency, trauma, malignancies, HIV/AIDS, cardiopathy), previous antibiotic treatment, clinical signs, treatment of the current episode, and mortality.

S. pneumoniae was identified by standard methods in the microbiology laboratory of each hospital. Isolates were serotyped at the National Pneumococcal Reference Center by means of the capsular swelling method with commercial antisera (Statens Serum Institut, Copenhagen, Denmark). During the study period, no attempt was made to distinguish between serotypes 6A and 6C, which were reported as type 6A/6C. Penicillin G and cefotaxime or ceftriaxone MICs were determined by the agar dilution method as previously described, and isolates were classified as susceptible, intermediate, or fully resistant according to the Clinical and Laboratory Standards Institute breakpoints.

The exhaustiveness of the surveillance system was evaluated by capture-recapture analysis of the 3 French national pneumococcal meningitis surveillance systems, and was estimated to be 61% (95% CI: 60%–66%) in 2001–2002. Quantitative data were analyzed with the Mann-Whitney U test and categorical data with the χ² test or Fisher exact test. The tests were 2-sided, the level of significance being set at P < 0.05. Statview 2 (Abacus Concepts) and Stata SE 9.1 software was used.

RESULTS

During the 8-year study period, 951 cases of pneumococcal meningitis were reported among 3312 children diagnosed with bacterial meningitis. Among the 951 pneumococcal meningitis, CSF Gram staining and culture were positive in 888/890 (90.8%) and 887/951 (93.3%) cases, respectively, and soluble antigen testing and CSF PCR were positive in 391 and 48 patients, respectively. Blood culture was positive in 75.4% of cases. When CSF culture was negative, microbiologic diagnosis was based on at least one of the following methods: CSF PCR.
TABLE 1. Characteristics of Pneumococcal Meningitis and Age Distribution From 2001 to 2008

<table>
<thead>
<tr>
<th>Year</th>
<th>N (%)</th>
<th>P</th>
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<tbody>
<tr>
<td>2001</td>
<td>153</td>
<td></td>
</tr>
<tr>
<td>2002</td>
<td>136</td>
<td></td>
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<tr>
<td>2003</td>
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<td>2004</td>
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<td>2005</td>
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<tr>
<td>2006</td>
<td>155</td>
<td></td>
</tr>
<tr>
<td>2007</td>
<td>152</td>
<td></td>
</tr>
<tr>
<td>2008</td>
<td>152</td>
<td></td>
</tr>
</tbody>
</table>

- No. pediatric wards
- Reporting bacterial meningitis
- Declaring no cases
- Nonresponder wards
- Pneumococcal meningitis
- Age (yr) median
- Underlying conditions

- Mean ± SD
- <2 yr, n = 607 (63.8)
- ≥2 yr, n = 344 (36.2)
- Case fatality rate

*Year-by-year comparison, 2001 to 2008 (ANOVA for mean age comparison).

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a higher proportion of pneumococcal meningitis in France (33%) than in the United States (23%).2,19,20 The relatively low vaccination uptake, together with the lack of a catch-up program, could have accelerated serotype replacement.29 The increase in the proportion of children with underlying conditions predisposing them to pneumococcal meningitis does not appear to explain our results (32.4% of children ≥2 years in 2001 vs. 30.5% in 2008; P = 0.9). Furthermore, the proportion of children with underlying conditions did not vary according to the serotype. Likewise, our results do not appear to be explained by changes in clinical practice after PCV7 introduction, as the indications for lumbar puncture did not change during the 5-year study period. However, a more thorough case reporting cannot be ruled out. Moreover, our findings may have been influenced by natural fluctuations (secular trends) in the incidence of pneumococcal meningitis and in the serotype distribution.

Our findings underline the complexity of pneumococcal ecology, with a noteworthy shift in the serotype distribution and in age of onset. They suggest that PCV13 could theoretically prevent 60% of cases of pneumococcal meningitis in France. Long-term surveillance of both IPD and carriage is crucial after PCV13 introduction.

ACKNOWLEDGMENTS

The list of acknowledged persons is available online (Acknowledgments, Supplemental Digital Content 2, http://links.lww.com/INF/A538).

REFERENCES


INCIDENCE OF INFLUENZA VIRUS INFECTION IN EARLY INFANCY

A PROSPECTIVE STUDY IN SOUTH ASIA

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Abstract: We evaluated infant serum from an immunization trial in Bangladesh to assess influenza hemagglutination inhibition antibody titer increases in 131 unimmunized infants from birth to 6 months. We detected 31 serologically defined infections. Combined with 10 additional rapid test-proven influenza cases, the minimal estimated incidence was 31 of 160 infants (19% CI: 24–41). These data suggest a high burden of influenza in young infants in tropical South Asia.

Key Words: influenza, infants

Accepted for publication August 5, 2010.

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The Mother’s gift project was supported by the Bill and Melinda Gates Foundation, United States Agency for International Development cooperative agreement HRN-A-0096-9006-00; Wyeth Pharmaceuticals Inc, the Thrasher Research Fund, Aventis Pasteur, ICDDR,B, and the Bloomberg School of Public Health at Johns Hopkins University. ICDDR,B acknowledges with gratitude the commitment of donors to the Centre’s research efforts. Ms. Henkle was supported by the Department of Health and Human Services, National Institutes of Health, National Eye Institute Training Grant Number EY 07127 Clinical Trials Training Program in Vision Research. Research support from Merck, the Bill and Melinda Gates Foundation, and USAID (to R.F.B.); research support from the Bill and Melinda Gates Foundation, USAID, the Thrasher Fund, Wyeth, Glaxo Smith-Kline, Sanofi-Aventis, and Merck, and lecture fees from GlaxoSmithKline and Sanofi-Aventis (to M.C.S.). No other potential conflict of interest relevant to this article was reported.

Registered with ClinicalTrials.gov: NCT 00142389.

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DOI: 10.1097/INF.0b013e3283186e39

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Young infants 0 to 6 months of age have high rates of hospitalization from influenza and its sequelae, as reported in high income regions. The cumulative incidence of influenza virus infection in 0 to 6 month old infants in the United States has been estimated as 12%. Historically, influenza has not been considered a frequent or serious illness of infants in the tropics. However, there is limited information on infection rates in infants in tropical regions, where influenza has a perennial pattern with year-round circulation of multiple subtypes and seasonal peaks in incidence. Recent clinic surveillance in Bangladesh reported that 10% of pneumonias in children <5 years of age are associated with influenza infection, but provided no information specific to young infants. Trivalent influenza vaccine is recommended in some high income regions for infants >6 months of age, but is not licensed for those <6 months.

The Mother's Gift study was a randomized maternal vaccine trial conducted in Dhaka, Bangladesh in 2004 and 2005. Maternal immunization with influenza vaccine reduced rapid test confirmed influenza infection by 63% and all febrile respiratory illness by 29% in infants 0 to 6 months of age. We analyzed prospectively collected infant sera in the Mother's Gift trial to estimate the incidence of serologically defined influenza virus infection in early infancy in Bangladesh.

MATERIALS AND METHODS

A full description of the Mother's Gift trial has been published, with a summary provided here. Three hundred forty pregnant women in Dhaka were randomized to receive polysaccharide pneumococcal vaccine (Pneumovax) or trivalent inactivated influenza vaccine (Fluarix). The influenza vaccine contained the World Health Organization (WHO) southern hemisphere recommendation for 2004: A/Fujian/41/2002-like (H3N2), A/New Caledonia/20/99-like (H1N1), and B/Hong Kong/334/2001-like strains. Of the 316 infants followed to 6 months of age in the trial, we report serologic values from the 157 infants of mothers who received pneumococcal vaccine. None of the mothers and infants included in this analysis received influenza vaccine.

From August 2004 through October 2005, cohorts of study mothers and their infants were followed from birth to 6 months by weekly home visit or telephone interview to record symptoms of febrile respiratory illness. Influenza A/B virologic testing in symptomatic infants was performed using a rapid detection test (RTD; Zstat, ZymeTs, Oklahoma City, OK). Influenza antibodies against the 3 vaccine strains were assayed using a standard hemagglutination inhibition (HAI) assay on infant sera collected at birth (zero), 10 weeks, and approximately 22 weeks of age.

We calculated the half-life of passively transferred HAI antibody, assuming exponential decay, among the infants from the full cohort with decreasing antibody values. We used the average calculated half-life of HAI antibody to estimate the expected antibody concentration at 10-week intervals. We defined serologic infection as a 4-fold increase in the observed titer compared with the expected declining titer, expressed as a later/earlier antibody ratio of ≥ 1 for sera obtained at least 10 weeks apart (Fig. 1). To account for the known variation in the HAI assay, we also specified that if the earlier titer was <1:10, the later titer should be ≥1:20. Incidence was calculated using Poisson regression with robust confidence intervals. The estimates are expressed as cumulative incidence per 100 infants and infections per 100 infant-months of observation.

The project protocol was approved by the Institutional Review Boards of International Centre for Diarrheal Disease Research, Bangladesh and the Bloomberg School of Public Health, Johns Hopkins University, and all mothers provided written informed consent.

![Figure 1](https://example.com/figure1.png)

**FIGURE 1.** Schematic of infant antibody dynamics, with and without influenza virus infection, showing that a 4-fold rise of antibody titer in infants over a 10-week period is equivalent to an observed antibody ratio of approximately 1.
RESULTS

The HAI data were available from 92% of 157 study infants. There was no difference in baseline demographic or birth characteristics comparing infants with and without samples available for analysis.

The estimated half-life of HAI antibody ranged from 33 to 45 days for the 3 virus subtypes in the 2 infant age groups. The overall weighted average half-life was 38 days, weighted by the number of sera tested for each influenza antigen. Thus, it would take about 76 days or 10.5 weeks for antibody titers to decline 2 half-lives (a decrease to 25% of the initial value). An antibody ratio of $\leq 1$ during a 10 week interval is approximately equivalent to a $\geq 4$-fold increase over the expected declining titer.

For the serologically defined incidence analysis, we excluded 14 (9.7%) infants because the planned study 22-week samples were collected at $\leq 28$ weeks of age. Among the evaluated 131 infants, 29 experienced 31 serologically defined influenza virus infections with vaccine subtypes (25.7/100 infants, 95% CI: 17.0–30.6), of which 6 (4.6/100 infants) had an absolute 4-fold increase (Table 1). Significantly more infections occurred after 10 weeks than before; 25 versus 6, respectively ($P < 0.001$). Two infants had 2 infections each with different subtypes, all between 10 and 22 weeks. The serologically defined incidence of influenza was 4.2 infections per 100 infant-months (95% CI: 3.0–5.9). Serologically defined infections were primarily A/Fujian (29 infections, 93%), with one each of A/New Caledonia and B/Hong Kong subtypes.

Our original report described 16 RDT cases (10.2%) in the 157 infants. Among the 131 included in this report, 15 (11.5%) had a positive RDT. Of these, 5 (33%) had concurrent serologically defined A/Fujian subtype infections that were considered the same infection. One infant had 2 infections: a serologically defined infection before 10 weeks of age plus an RDT-proven infection at 22 weeks of age. Adding the 10 distinct RDT-proven infections resulted in a total of 41 influenza infections and a cumulative incidence of 31.3 of 100 0 to 6 month old infants (95% CI: 23.6–41.4). The overall incidence rate of infection in 0 to 6 month old infants was 5.6 of 100 infant-months (95% CI: 4.2–7.4) (Table 1).

We explored the relationships between serologically defined influenza virus infection, RDT-proven influenza, and reported respiratory illness with fever. Of 12 infants with RDT-proven influenza by 22 weeks, 4 (25%) had serologic evidence of infection. Including data up to 22 weeks, 75.9% of 29 infants with serologically defined influenza virus infection reported any respiratory illness with fever compared with 52.0% of 102 with no serologically defined infection ($P = 0.02$).

DISCUSSION

We are not aware of a published, prospective serologic study of influenza incidence rates in young infants in Asia. Our unique prospective serologic and RDT data show a substantial burden of influenza in this region in young infants. We found that 31 of 100 (95% CI: 23.6–41.4) infants at 0 to 6 months were infected over a 15-month calendar period. Three infants (2.3%) each had 2 separate influenza virus infections detected before 6 months of age.

By comparison, in the United States, Glezen et al found an average annual influenza incidence of 12.4 of 100 infants aged 0 to 6 months, using culture and serology in Houston from 1975 to 1985. Since they only assessed antibodies to the 2 predominant influenza virus strains each year, this is likely an underestimate. Infection in the Texas study was often asymptomatic, similar to our study. Both observations suggest that studies relying on symptoms or clinic visits of young infants as indicators for influenza testing will miss a significant proportion of infections in these infants. Our data in young infants suggests that prospective serology more than doubles the number of influenza virus infections compared with clinic surveillance of febrile illness (Table 1).

A similar serologically defined infection methodology was used to assess H1N1 influenza virus infection by Reuman et al in a natural history study of 18 US infants followed until age 3 to 6 months during the 1978–1979 season. A 4-fold rise over expected titer was seen in 28% of 18 infants of nonimmune mothers, similar to our serologically defined incidence of 24%. The 33 to 45 day half-life of maternally acquired infant antibodies we observed was similar to a US maternal immunization study reporting infant influenza antibody half-lives of 40 to 50 days, using a non-HAI ELISA assay.

One of the strengths of this serologic analysis was the careful follow-up and sampling of nearly all infants in the study cohort, which allowed detection of a substantial number of asymptomatic influenza virus infections. However, the 10+ week interval between sera tested for HAI antibody results made it challenging to associate serologically defined infection with the weekly reported symptoms and illness. These serologic data are a minimal estimate for several reasons. We only tested for antibodies to the 3 influenza vaccine subtypes and did not assess infection with other influenza subtypes. Further, some infants with higher maternal antibody concentration may have had an attenuated antibody response to infection. Finally, the RDT test that we used has low sensitivity, suggesting that at least 30% of true cases were missed. Both these factors indicate these data are a minimal estimate of the true rate of infection. The discrepancy between RDT and serolog-
ically detected influenza cases likely represents infections with influenza subtypes not included in the H1N1 serologic testing panel. Young infants are a high-risk group for influenza infection, illnesses, and sequelae which can lead to substantial parent/caretaker absenteeism. We observed a minimal influenza virus infection rate of 31 of 100 (95% CI: 23.6–41.4), which suggests a sizable burden of influenza illness in this tropical setting. These data are part of a growing body of literature that suggests influenza illness in infants and children may also be a neglected tropical disease.  

Infants in tropical and subtropical regions comprise more than 60% of the 136 million annual global births, and many are exposed to influenza virus infection year-round. Additional studies of the incidence of influenza in young infants in Asian and other tropical regions should be carried out to better define the burden of preventable influenza illness in this vulnerable group. Maternal antenatal immunization with influenza vaccine is a proven and implementable strategy to protect both pregnant women and their young infants in tropical regions.

ACKNOWLEDGMENTS

The authors thank Dr. David Sack for his invaluable assistance in the project. The authors also thank the ICDDR,B field and data management team for their efforts, and they express their gratitude to the families who participated in the project. Special thanks to Justin Lessler for figure graphics.

Dr. Steinhoff had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Steinhoff, Zaman, Roy, Arifeen, Breiman. Acquisition of data: Roy, Zaman, Arifeen, Ragib, McNeal. Analysis and interpretation of data: Henkle, Steinhoff, Zaman, Roy, Alam, Omer, Moss, Arifeen. Drafting of the manuscript: Henkle, Steinhoff, Moss. Critical revision of manuscript for important intellectual content: Steinhoff, Omer, Moss, Zaman, Roy, Breiman, Ragib, McNeal. Statistical analysis: Henkle, Steinhoff. Obtaining funding: Steinhoff. Administrative, technical, or material support: Arifeen, Zaman, Steinhoff, McNeal. Study supervision: Zaman, Steinhoff.

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In this case series, data on all patients within the cohort consecutively diagnosed with 2009 H1N1 influenza between August 16 (week 33) and December 31, 2009 (week 50) were presented. The first pandemic wave in Spain occurred between weeks 38 and 49. In our geographic area, the incidence of 2009 H1N1 influenza among children aged 5 to 14 years reached its peak between weeks 43 and 46 (1200 infections per 100,000 inhabitants). H1N1 influenza infection was confirmed by means of real-time reverse-transcription-polymerase chain reaction on nasopharyngeal swab samples. As per national guidelines, hospital admission was mandatory in patients with CD4 cell counts <350 cells/mm³; for the remaining, clinical management was individualized at the discretion of the treating physician. Epidemiologic, clinical, and outcome characteristics of H1N1 influenza patients were summarized, and the main clinical and immunologic HIV-related variables were compared with those of the rest of the cohort. Fisher exact test and Mann-Whitney U tests were used to compare proportions and continuous variables, respectively. Other nonparametric tests were used as indicated. A P < 0.05 was considered statistically significant.

RESULTS

Thirteen HIV-infected patients (5 females, median age: 11.7 years, 12 of them vertically infected; Table, Supplemental Digital Content 1, http://links.lww.com/INF/A589) were diagnosed with H1N1 influenza during the 2009 season. Within the cohort (attack rate: 7.4%, 95% confidence interval: 0.65%-14.2%). The main HIV-related characteristics were not different from those of the rest of the cohort (gender, age, acquired immunodeficiency syndrome [AIDS] diagnosis, nadir CD4 cell count, and 2009 second semester CD4 cell count and HIV plasma viral load; data not shown). No patient had received the pandemic influenza vaccine, which was not available in Spain till November 16 (week 46). Regarding HIV infection, 3 patients had previously developed AIDS (including 2 cases of HIV-related progressive encephalopathy, patients 2 and 8), and the median nadir CD4 cell count was 434 cells/mm³ (range: 44-2340 cells/mm³). Patient 13 was affected with insulin-dependent diabetes mellitus; no other comorbidities were observed. At the time of influenza, 12 of 13 patients were receiving combined antiretroviral treatment, with optimal virologic response in most of them (9/12, less than 50 to 100 RNA-HIV copies/mL, depending on the center) and absolute CD4 cell counts within normal ranges in 10 patients. Median time between the onset of symptoms and first medical attention was 2 days. All patients developed high-grade fever for a median time of 3 days. Cough (n = 12), rhinorrhea (n = 11), headache (n = 6), and myalgia (n = 4) were the most common complaints, together with diarrhea and vomiting (2 patients each), and odynophagia, anorexia, and conjunctival inflammation (1 each). Most patients (11/13) received a 5-day course of either oseltamivir or zanamivir, which were initiated empirically and not prescribed on microbiologic confirmation. Antivirals were not prescribed to patients 9 and 11 because fever had ceased by the time they were first seen. Eleven patients were managed on an outpatient basis. Patient 13 was admitted as a precaution because of her risk factors (moderate immunosuppression and diabetes) and was discharged 3 days later without incidents. Patient 5 was admitted because of severe immunosuppression and diagnosis of pulmonary bacterial coinfection without hypoxemia; he received both antiviral therapy (oseltamivir) and a 10-day course of sequential treatment with intravenous cefotaxime and oral levofloxacin, and was discharged without incident on day 4. Patient 4 also received a 10-day course of oral amoxicillin-clavulanate because of a diagnosis of retrocardiac pneumonia. The 2 cases with presumed bacterial pneumonia were not microbiologically confirmed. All patients recovered uneventfully within a median time of 7 days after onset of symptoms.

Previous AIDS diagnosis (P = 0.58), comorbidities (P = 0.58), and nadir (P = 0.39) and current (P = 0.31) CD4 cell count were not associated with a more severe evolution of 2009 H1N1 influenza (patients requiring hospitalization or antibiotics). Similarity, H1N1 infection had no effect on CD4 cell count (median values: 730 cells/mm³ before influenza A vs 838 cells/mm³ in the following control after 6.4 weeks, median time; P = 0.31) or HIV viral load (1.7 vs. 1.9 log RNA-HIV copies/mL; P = 0.12) evolution.

DISCUSSION

Pandemic influenza A (H1N1) virus emerged in Mexico in March 2009 and caused extensive disease worldwide, especially in children and young adults. H1N1-infected pediatric patients represent a highly susceptible and vulnerable population for whom the investigation of pandemic influenza is mandatory. Although most pediatric illnesses are acute and self-limited, the pandemic led to an increased burden of care and associated higher hospitalization, morbidity and mortality rates than seasonal influenza in previous years. Among pediatric patients, those aged <2 years and children with one or more pre-existing conditions were at higher risk for severe illness, hospitalization, and death. In the largest case series reported to date, the majority of hospitalizations occurred in infants affected with hypoxemia, 20% of whom required admission to an intensive care unit and mechanical ventilation, rates of confirmed secondary bacterial coinfection were low. Previous underlying conditions affected 30% to 60% of hospitalized children in different series, with asthma and other chronic pulmonary diseases, neurologic disorders, and heart disease being the most common. Despite previous immunosuppressing conditions affecting approximately 10% of patients requiring admission in different adult and pediatric series specific features for HIV infection were not reported; in the study by Libster et al., HIV-infected children were less likely to be admitted to an intensive care unit than patients with other comorbidities.

In our series, the presenting features of 2009 H1N1 influenza were similar to those described in the general pediatric population. An influenza-like illness was present in all cases (fever, cough, and/or rhinorrhea), and gastrointestinal symptoms affected one-third of the patients. Likewise, bacterial coinfection was suspected in only 2 patients but was not confirmed. Notably, 3 children presented other comorbidities, and CD4 cell counts were within the ranges of moderate or severe immunosuppression in 3 patients at presentation. These findings were not associated with a worse outcome, in contrast with a recent series of H1N1 influenza in 10 children with acute lymphoblastic leukemia, in whom intensity of immunosuppression correlated with disease severity. As recommended, antivirals were prescribed in most cases, and in 7 of 11 children within 48 hours of onset. No relevant adverse effects were noted. Whether early antiviral therapy decreased the severity of the disease or led to a prompt clearance of the 2009 H1N1 virus from the upper respiratory tract remains unknown. Two patients-overcame influenza A without the need for antivirals. As in the adult HIV-infected population, H1N1 influenza did not seem to have a major impact on HIV infection control in our series, considering the evolution of CD4 cell counts and HIV viremia.

In our series, the attack rate of influenza A among HIV-infected children was 7.4%, lower than the 22% reported by Antonietti et al. (11/49 HIV-infected pediatric patients in Campania, Italy). This is surprising considering the high attack rate of H1N1 infection in adults (approximately 15%) and in the pandemic age (up to 20%) and the better ascertainment of influenza A in
the HIV-infected population, and may be partially explained by a potential under diagnosis bias in patients with mild disease. Small sample size, lack of standardization in clinical evaluation, and absence of a control group are other obvious limitations of our observational study. However, our results are consistent with those of studies involving adult HIV-infected populations and suggest that HIV infection may not represent a major risk factor for severe influenza A (H1N1) infection in the pediatric age. Nevertheless, until larger studies are available, HIV-infected children should still be considered to be at high risk and promotion of H1N1 vaccination, early case recognition and empiric therapy are recommended.

APPENDIX

CORSIFE-2 Cohort consists of HIV-infected pediatric patients recruited by Hospital Sant Joan de Déu; Claudia Fortuny, Antoni Noguera-Julian, Rosa Pino, Carmen Muñoz-Almagro; Hospital Materno-Infantil Vall d’Hebron; Maria Concepción Figueras, Andrea Martin, Pere Soler-Palacín, Maria Espuña; Hospital del Mar: Antoni Mur, Núria López; Hospital Germans Trias i Pujol: María Méndez, Carlos Rodrigo; Hospital Arnau de Vilanova: Teresa Vallmanya; Hospital General de Granollers: Maria Teresa Coll; Hospital de Mataró: Lourdes Garcia; Corporación Sanitaria Parque Tauli: Valentí Pineda; Hospital Joan XXIII: Antoni Soriano; Hospital Sant Joan de Reus: Joaquim Escriva; Hospital Sant Joan de Déu de Manresa: Núria Rovira; Hospital Son Dureta: Joaquín Duñach; Hospital Clinic-IDIBAPS, University of Barcelona, Iñaki Pérez.

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SEVERE OCULAR REACTIONS AFTER NEONATAL OCULAR PROPHYLAXIS WITH GENTAMICIN OPHTHALMIC OINTMENT

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Abstract: In this study, we report 4 infants who developed severe ocular reactions after neonatal ocular prophylaxis with gentamicin ophthalmic ointment during a period of erythromycin ophthalmic ointment shortage. In light of this experience, gentamicin ophthalmic ointment should not be used as an alternative for neonatal ocular prophylaxis.

Key Words: ocular prophylaxis, gonococcal ophthalmia neonatorum, gentamicin ophthalmic ointment, erythromycin ophthalmic ointment shortage

Accepted for publication July 27, 2010.

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DOI: 10.1097/INF.0b013e31818e2e25

Ophthalmia neonatorum is defined as conjunctivitis that occurs within the first 28 days of life. Gonococcal conjunctivitis in the newborn can be prevented by instilling a prophylactic agent into the eyes just after delivery; neonatal ocular prophylaxis is mandated by state law in most of the United States. Three agents have established efficacy and safety for this purpose: 0.5% erythromycin ointment, 1% tetracycline, and 1% silver nitrate solution. The only prophylaxis available for ocular prophylaxis that is currently available for use in the United States is 0.5% erythromycin ointment.

In August 2009, the Centers for Disease Control and Prevention (CDC) informed physicians of a shortage of 0.5% erythromycin ointment because of a change in manufacturers. A set of interim guidelines was provided but the alternative agents listed, including azithromycin and gentamicin ophthalmic preparations, are not FDA-approved for this indication, and there are no clinical data on efficacy for neonatal ocular prophylaxis.1 Azithromycin is only available as an ophthalmic solution and must be given as drops; it is also more expensive than other ophthalmic antibiotics. In October 2009, the CDC released a report of lid swelling and dermatitis associated with the use of 0.3% gentamicin ointment.1

In this article, we report 4 infants at our institution who developed ocular reactions after neonatal ocular prophylaxis with gentamicin ophthalmic ointment.

CASE REPORT

A baby boy was born to a 22-year-old mother at 40 weeks gestation via normal spontaneous vaginal delivery. The mother had
received routine prenatal care and her pregnancy was uneventful. Maternal screening for Chlamydia trachomatis and Neisseria gonorrhoeae performed 8 weeks before delivery were negative. On the second day of life, the baby was noted to have bilateral copious green eye discharge with severe eyelid erythema and swelling. A small blister was also found under the right eye. The patient was feeding well, had a strong cry, and remained afebrile. A presumptive diagnosis of preseptal cellulitis was made; a full septic work-up was done and antibiotics were started. Gram stain of ocular discharge and cultures for N. gonorrhoeae and herpes simplex virus were negative. Blood and CSF cultures were also negative. A review of labor and delivery management revealed that the baby had received gentamicin (0.3%) ophthalmic ointment in place of erythromycin (0.5%) ophthalmic ointment for ocular prophylaxis. Antibiotics were stopped on the fourth day of life and symptoms completely resolved by the seventh day of life.

During the next 3 weeks, 3 more infants who had received gentamicin (0.3%) ophthalmic ointment developed less severe but similar findings of eye discharge, blistering, eyelid erythema, and swelling within 48 hours of delivery. These infants were managed without an infectious work-up and no systemic antibiotics were given. All infants went home without complication.

Further investigation revealed that gentamicin ophthalmic ointment was being provided to the delivery room for neonatal ocular prophylaxis between October 1, 2009 and December 21, 2009. A total of 662 infants received ocular prophylaxis with gentamicin during this period. The incidence of ocular reaction at our institution was 0.6 per 100 newborns.

**DISCUSSION**

Prophylaxis for opthalmia neonatorum is a common practice that dates back to the late 1800s.7 Neonatal ocular prophylaxis will prevent gonococcal ophthalmia; however, it is not effective for the prevention of chlamydial ophthalmia. The frequency of gonococcal ophthalmia is largely related to the prevalence of maternal genital infection with N. gonorrhoeae. Routine screening and treatment of pregnant women for gonorrhea probably has more of an effect on prevention of gonococcal ophthalmia in the United States today. We identified 2 other reports in the literature of similar reactions to gentamicin ophthalmic ointment in neonates.6,8 In 1991, 71 infants born at an Israeli hospital received ocular prophylaxis with gentamicin sulfate ophthalmic ointment because of a temporary shortage of tetracycline ophthalmic ointment.4 Five (7%) of these infants developed ocular reactions similar to those observed in our infants, which resolved within 2 to 3 weeks of onset. Two hospitals in Philadelphia recently reported a series of 26 newborns (rate 5.6/100 newborns) with periorbital ulcerative dermatitis after changing to gentamicin ointment in October 2009 during the erythromycin shortage.3 In light of these reports and our experience, gentamicin ophthalmic ointment should not be used as an alternative for neonatal ocular prophylaxis.

The mechanism of the reaction to gentamicin is unclear. It may be due to the vaso-occlusive effects of gentamicin on the thin skin of the newborn eyelid.5 Some also postulate that the reaction may be due to the contact dermatitis, either from the gentamicin, or a preservative, such as paraben, used to make the preparation, or a combination of both.3 The other reports also noted a relationship between the amount of ointment left on the skin and the severity of the reaction; the rash was milder if excess ointment was wiped away after application.1,5

Erythromycin (0.5%) ophthalmic ointment is currently the only available preparation for neonatal ocular prophylaxis in the United States. Using other available antibiotic ophthalmic preparations has the risk of adverse events as these agents have not been well studied for this use in this age group. These cases serve as a reminder to clinicians to consider drug reaction in the differential when using an agent that is not part of standard practice. Because this reaction is not a common finding, one infant was subjected to unnecessary invasive procedures such as a lumbar puncture. The upcoming 2010 CDC Sexually Transmitted Diseases Treatment Guidelines recommend that if erythromycin ophthalmic ointment is not available, infants deemed to be at risk for exposure to N. gonorrhoeae, that is, the mother has untreated gonorrhea or a history of inadequately gonorrhea during pregnancy, the infant should be treated for presumptive infection as currently recommended, with either ceftriaxone (25-50 mg/kg, intravenously or intramuscularly, not to exceed 125 mg, in a single dose) or cefotaxime (100 mg/kg, intravenously or intramuscularly in a single dose).6

The shortage of erythromycin ophthalmic ointment and lack of good alternative agents are an indication for further studies in the area of neonatal ocular prophylaxis. Both gentamicin and azithromycin are active in vitro against N. gonorrhoeae, but there are few studies to support their effectiveness for treatment of gonococcal infections in the clinical setting.7,8 This may be the time to re-examine the issue to be better prepared, should another shortage occur in the future.

**REFERENCES**


**SEVERE GENITAL ULCERATION IN AN ACUTE EPSITINE-BARR VIRUS INFECTION**

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Abstract: Genital ulceration is a rare manifestation of infectious mononucleosis caused by Epstein-Barr virus (EBV). We report a girl with severe genital ulceration and tissue necrosis during primary EBV infection that required surgical debridement. The excised genital tissue was EBV positive in reaction—positive whereas in situ hybridization was negative. This suggests that the ulceration was likely because of the inflammatory response to the virus and not because of the intense viral infection of the genitalia.

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Key Words: Epstein-Barr virus (EBV), genital ulceration
Accepted for publication July 27, 2010.
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Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the Journal's Web site (www.pidj.com).
DOI: 10.1097/INF.0b013e3181f41b2c

Epstein-Barr virus (EBV) is a widely distributed herpes virus. It is transmitted by intimate contact with body secretions, especially saliva. EBV infection of mononucleosis (EBV-IM) is primarily a disease of children, adolescents, and young adults. The establishment of IM as a clinical entity is credited to Sprunt and Evans, who in 1929 described 6 cases of fever, lymphadenopathy, and prostration occurring in previously healthy young adults. Patients with IM usually present with fever, pharyngitis, and lymphadenopathy. However, EBV can affect virtually any organ system and has been associated with a wide range of clinical presentations. Genital ulceration is a rare manifestation of acute EBV infection, described by Brown and Stenchever in 1977. We report a case of severe genital ulceration in a primary EBV infection that caused a necrotizing soft tissue infection. The surgically debrided tissue provided an opportunity to determine if EBV was present in the genital lesion.

CASE REPORT

A previously healthy 14-year-old girl presented to the emergency department with a 1-day history of fever, sore throat, and generalized weakness, followed by perianal pain, itching, and dysuria. She had her menstrual cycle 1 day earlier. There was no history of intercourse or orogenital contact, but she admitted to having a hand-genital contact 1 month before.

On examination, she looked uncomfortable with a temperature of 40.5°C, pulse rate of 130/min, and blood pressure of 153/75 mm Hg. An exudative membrane covered the tonsils, with no associated cervical lymphadenopathy. Examination of the genitalia showed bilateral ulcerated lesions with necrotic bases involving the labia minora mainly on the right side. The lesions were surrounded by indurated erythematous area over the vulva (Fig. 1, Supplemental Digital Content 1, http://links.lww.com/INF/A582). There was no inguinal lymphadenopathy and the rest of the examination was unremarkable.

The white blood cell count was 2.86 × 10^9/L, and lymphocyte count was 1.745 × 10^9/L. Atypical lymphocytes were present at 0.200 × 10^9/L. A computed tomography scan of the abdomen and pelvis showed no evidence of deep pelvic extension of the necrotizing soft tissue infection. Being allergic to penicillin, she was treated immediately with intravenous vancomycin (1 g intravenous [IV] every 8 hours), cefazolin (800 mg IV every 8 hours), and gentamicin (100 mg IV every 8 hours). She was quickly taken to the operating room for debridement of the necrotic tissue in the perineum. Gram stain of the excised tissue showed +1 polymorphonuclear cell, +1 Gram-positive bacilli, and occasional Gram-negative bacilli. On culture, growth of Lactobacilli and Escherichia coli were noted. Other microbiologic cultures were negative including a throat swab and multiple sets of blood culture. Herpes simplex virus IgM and IgG serologies were negative. A heterophile antibody test and EBV VCA IgM were positive whereas EBV VCA IgG was nonreactive. Histopathologic examination of the excised lesion showed acute and chronic inflammation with Gram-positive bacilli and fibrin thrombi occluding many of the blood vessels.

In an attempt to define the link between EBV and the genital lesion, an EBV polymerase chain reaction (PCR) test was performed on the serum and the genital tissue and both were positive. EBV in serum was detected at less than 100 copies per ml by semi-quantitative PCR. However, in situ hybridization for detecting EBV in the tissue was negative.

DISCUSSION

Acute genital ulceration in adolescents, with no documented infectious etiology, was first described in 1913 by Lipschutz. Since then, several reports were published describing the possible infectious causes of such lesions including EBV. The majority of reported cases associated with EBV-IM have been in young females. However, a solitary penile ulcer associated with IM was reported by Lawwe and Shaw in 1983.

Genital ulceration in acute EBV-IM is a rare clinical entity of an undefined pathogenesis. Reported attempts to identify EBV from these lesions were variably positive, negative or not attempted.

It was previously suggested that the genital lesion results from direct inoculation of the virus. However, other reports suggested that nonsexual modes of transmission, such as vesicles or direct self inoculation, can produce genital ulcers.

In the case presented here, we attempted to define the mechanism by which the genital lesion developed. EBV PCR testing of the serum and the excised genital tissue was done simultaneously, and both were positive. However, the tissue could have been falsely positive from blood present in it, and to minimize that, EBV in situ hybridization was performed. It is based on maintaining the structural integrity of the tissue while allowing the nucleic acid of the pathogen to be released and denatured to a single strand with the base sequence intact as described previously. Nevertheless, the EBV in situ hybridization was negative. This result is consistent with previous reports, suggesting that the development of genital lesions in EBV-IM is not the result of large amounts of virus at the site of the ulcer but is more likely to be an immunologically driven inflammatory consequence. The bacterial infection was thought to be secondary to the ulcerative process but may have contributed to the tissue necrosis.

ACKNOWLEDGMENTS

The authors thank Dr. Gareth Jevon for performing the in situ hybridization test and Dr. Edith Blondeel-Hill for her expert involvement in the diagnosis of this case.

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INVASIVE ASPERGILLOSIS IN CYSTIC FIBROSIS

A FATAL CASE IN AN ADOLESCENT AND REVIEW OF THE LITERATURE

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Abstract: Invasive aspergillosis is a rare complication of cystic fibrosis. In this article, we describe a case of an adolescent with cystic fibrosis, which was well-controlled previously, colonized with *Aspergillus fumigatus*. The patient developed fatal disseminated aspergillosis in the absence of any preexisting risk factors after a short course of intravenous corticosteroid treatment.

Key Words: invasive aspergillosis, cystic fibrosis, fatal, adolescent

Accepted for publication July 27, 2010.

Invasive aspergillosis occurs most commonly in patients with immunodeficiencies, primarily neutropenia or corticosteroid-induced immunosuppression. Although up to 60% of patients with cystic fibrosis (CF) have *Aspergillus* species in their sputum, disseminated invasive aspergillosis is exceedingly rare in cases of CF. In this article, we describe a case of an adolescent with CF, which was well-controlled previously, colonized with *Aspergillus fumigatus*. The patient developed fatal disseminated aspergillosis in the absence of any preexisting risk factors after a short course of intravenous (IV) corticosteroid treatment.

CASE REPORT

The patient was a 15-year-old boy diagnosed with CF at the age of 3 weeks after presenting with failure to thrive. He had pancreatic insufficiency and a baseline forced expiratory volume in 1 second of 60% predicted. In the 4 years before this admission, he had 2 acute pulmonary exacerbations requiring hospitalization for IV antibiotics. He played soccer and had no limitations on his activities. He lived in a rural area and at the time of his illness, there were extensive renovations occurring at his high school. He had no history of allergic bronchopulmonary aspergillosis (ABPA) or of CF-related diabetes. Previous sputum culture tests had shown growth of *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, and *A. fumigatus* (first cultural examination was positive in August 2000). His regular medications included inhaled tobramycin 300 mg twice a day every other month, DNase, a proton pump inhibitor, digestive enzymes, and vitamin supplements. He had not been on any immunomodulatory agents such as intral iv corticosteroids, high-dose ibuprofen, or azithromycin.

In January 2008, he was admitted to the local hospital with chest pain, increasing cough, dysnea, fever, and rust-colored sputum. On examination, his oxygen saturation was 86% in room air, respiratory rate 24 to 35 breaths per minute, and heart rate 124 beats per minute. His weight was 55 kg. On chest auscultation, there was decreased air entry bilaterally, but no wheezing. Complete blood count demonstrated a white blood cell count of 13.7 × 10^9/L with neutrophils of 9.4 × 10^9/L, hemoglobin of 158 g/L, and platelet count of 246 × 10^9/L. Erythrocyte sedimentation rate was 63 mm/h and total serum IgE was 81 IU/mL. Chest radiograph revealed patchy bilateral infiltrates and subcutaneous emphysema. The patient was admitted to the local hospital and treated with 4 L per minute of supplemental oxygen through nasal prongs to maintain his oxygen saturation above 93%. IV tobramycin, cefazidine, and levofloxacin were administered on the basis of the results of his most recent sputum cultures.

On the second day of hospitalization, his respiratory status deteriorated with worsening of dyspnea, increased work of breathing, and increased oxygen requirement to 57% IV methylprednisolone (60 mg every 6 hours) was started at this time and continued for 5 days after which the dose was reduced to half (30 mg IV 6 hours) for the following 5 days. The patient remained in stable condition until day 8 of hospitalization when he developed a pneumothorax for which a chest tube was placed. IV trimethoprim-sulfamethoxazole and cloxacillin were empirically added to his antibiotic regimen. The patient then had a further episode of hypotension which was treated with IV fluid boluses. Amphotericin B (60 mg IV daily) was empirically started. The following day the patient was referred to our institution and transferred to the critical care unit for further management.

On arrival to the critical care unit, the patient's oxygen saturation was 92% to 95% on 15 L/min high flow oxygen by face mask. Air entry to the right upper lobe was markedly reduced and there were crackles throughout the right lung on auscultation. His chest wall and shoulders were tender on palpation. His chest radiograph revealed diffuse, bilateral infiltrates. IV tobramycin, cefazidine, levofloxacin, trimethoprim-sulfamethoxazole, and cloxacillin were continued, but the amphotericin B was stopped, as there was no clinical evidence of invasive fungal infection. On day 9 of hospitalization, the patient's respiratory condition worsened with increased work of breathing and tachypnea with a rise in his venous PCO2 to 65 mm Hg and an increased oxygen requirement of 100% by face mask. He was producing copious amounts of reddish-brown sputum. He was supported with noninvasive positive pressure ventilation. Blood cultures were negative for bacterial growth. Expected sputum culture from day 9 of hospitalization was positive for *S multivialis* and heavy growth of *A. fumigatus*. On day 10 of hospitalization, he was intubated because of hypoxemia and hypercarbia despite maximal noninvasive positive pressure ventilation support. Because of his deteriorating condition, antimicrobial therapy was empirically changed to IV meropenem, ciprofloxacin, trimethoprim-sulfamethoxazole, vancomycin, liposomal amphotericin B (300 mg IV daily), and osemulvir. Respiratory specimens were negative for *parainfluenza*, adenovirus, respiratory syncytial virus, human metapneumovirus, and influenza A and B viruses by immunofluorescence. On day 11 of hospitalization, the patient became hypotensive with mean blood pressures of 55 to 65 mm Hg and had poor peripheral perfusion. He required multiple IV fluid boluses as well as IV albunin, dopamine, epinephrine, norepinephrine, and hydrocortisone. He died on day 11 of hospitalization with refractory shock, hypoxemia, and profound hypercarbia. Consent for autopsy was obtained from the patient's parents.

Postmortem examination revealed extensive, necrotizing pneumonia with hemorrhagic consolidation. Microscopic examination of the lung demonstrated fungal hyphae with intravascular...
invasion and culture tests of lung tissue were positive for *A. fumigatus*. There were fungal microabscesses with few neutrophils and no fibrosis in the myocardium, adrenal gland, kidney, spleen, central nervous system, and pulmonary hilar nodes.

**DISCUSSION**

*Aspergillus* species are saprophytic fungi that are present throughout the environment, particularly in soil and decaying organic matter. Aspergilli commonly colonize the lungs of patients with underlying bronchiectasis, including up to 60% of those with CF. ABPA is the most common *Aspergillus*-associated form of lung disease in CF. Aspergillosis may develop, and in most cases, represent colonization, although localized invasion with pulmonary hemorrhage can occur. However, disseminated invasive aspergillosis, in the absence of immunosuppression secondary to lung transplantation, for example, occurs rarely in CF.

To our knowledge, there are 3 previously reported cases of disseminated invasive aspergillosis in CF patients (Table 1). In all 3 cases, the diagnosis was made late in the clinical course or post-mortem, and consequently, they had received limited or no IV antifungal treatment. All 3 patients were adolescents or young adults and had a fatal outcome. Two were previously colonized with *A. fumigatus*, but only one had a history of ABPA. Oral and/or inhaled corticosteroids may have predisposed 2 of them to developing invasive aspergillosis. In the case reported by Brown et al, the use of azathioprine likely contributed to suppression of cellular immune function. In the third case, viral induced lymphocytopenia in a previous smoker was identified as the only potential predisposing factor. Our patient was well-nourished, had no history of CF-related diabetes, and a relatively good pulmonary function. The use of IV methylprednisolone therapy during his hospitalization was the only identifiable factor predisposing him to invasive aspergillosis.

High doses and prolonged courses of corticosteroids have been linked with an increased risk of and adverse outcome in invasive aspergillosis. However, there is no clearly defined threshold dose or duration that can be used to identify patients who are at risk. On rare occasions, even short-term (1–2 weeks) IV corticosteroid treatment, at doses as low as the equivalent of 1 mg/kg/d of methylprednisolone, has been associated with the development of invasive aspergillosis, even in immunocompetent patients. Similarly, our patient received a relatively short course of IV methylprednisolone (5 mg/kg/d for 5 days followed by 2.5 mg/kg/d for another 5 days).

Although the risk of invasive aspergillosis increases with longer durations of corticosteroid therapy, the onset of the immunomodulatory effects of steroids can be relatively rapid. For example, corticosteroids have been shown to cause a redistribution of CD4+ and CD8+ T lymphocytes from the circulation within 4 hours of IV injection of hydrocortisone. Corticosteroids have also been noted to inhibit lipoxygenase-induced cytokine production by alveolar macrophages, the primary innate cellular defense against inhaled *Aspergillus* conidia, within 2 hours of exposure. Other in vitro experiments have demonstrated a >20% reduction in neutrophil-mediated damage to *A. fumigatus* hyphae within 10 minutes of exposure to low doses of dexamethasone. By 120 minutes of dexamethasone exposure, the polymorphonuclear-cytotoxic-mediated damage to *Aspergillus* hyphae can be almost completely abolished.

Our patient was previously colonized with *A. fumigatus* and likely had chronic pulmonary inflammation secondary to CF. Microscopic examination demonstrated diffuse necrotizing pneumonia secondary to *Aspergillus*, which may have been occurring for some time and could have contributed to the patient’s initial pulmonary deterioration. However, the small extrapulmonary microabscesses, with minimal inflammation and no significant fibrosis were more suggestive of an acute process. Short-term IV steroid therapy may have been sufficient to suppress immune function and allow hematogenous dissemination to occur.

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CUMULATIVE CARDIAC TOXICITY OF SODIUM STIBOGluconATE AND AMPHOTERICIN B IN TREATMENT OF KALA-AZAR

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Abstract: Kala-azar or visceral leishmaniasis is a disseminated protozoal infection caused by parasites of the genus Leishmania (Leishmania donovani in India). Conventional therapy for visceral leishmaniasis continues to be pentavalent antimony (sodium antimony gluconate [SAG]). Amphotericin B is widely used for SAG-unresponsive cases and sometimes even as a first-line drug, especially in endemic areas. With the conventional regimen of SAG, cardiac toxicity has been reported in 8% to 17% of cases with 5% to 7% of them having fatal toxicity. Cardiac toxicity is uncommon with amphotericin B with only few isolated reports in the literature.6–8 but significant cardiac toxicity may occur when amphotericin B is used in patients with pre-existing cardiovascular disease or electrolyte abnormalities.6–9 We report 3 patients with kala-azar in whom coadministration of SAG and amphotericin B led to sudden deaths and arrhythmia.

CASE 1

A 7-year-old boy, from Bihar, presented with fever for 4 months and progressive abdominal distension. There was no history of jaundice, joint pains, rashes, breathlessness, bleeding, or recurrent infections. On examination, he had pallor with massive hepatosplenomegaly. The remaining examination was unremarkable. Investigations revealed pancytopenia with hemoglobin of 8.3 g/dL, total leukocyte count of 2500/μL with neutrophils 35%, lymphocytes 60%, and platelet count of 88,000/μL. Bone marrow aspirate examination revealed amastigote forms of L. donovani, confirming the diagnosis of VL. Renal and liver function tests (RFT, LFT), serum electrolytes, electrocardiogram (ECG), and chest radiograph were normal. Treatment was initiated with injection of sodium stibogluconate 20 mg/kg/d intramuscularly once a day. Despite treatment, his general condition deteriorated and investigations revealed worsening pancytopenia. In view of these developments and the fact that he resided in North Bihar, an area with a known high prevalence of resistance to SAG, he received amphotericin B after 6 days, and SAG was discontinued. RFTs and serum electrolytes (sodium, potassium, and ionized calcium) were normal at that time. Amphotericin B was administered as a slow intravenous infusion during 4 hours in a dose of 0.25 mg/kg on the first day and increased to 0.5 mg/kg on the next day. After receiving 2 doses of amphotericin B, the boy developed sudden tachycardia and shock. An ECG recording at this time revealed ventricular tachycardia. Defibrillation was attempted and supportive measures were instituted, but the child could not be revived.

CASE 2

A 5-year-old male child from Bihar was diagnosed as having VL and was treated with intramuscular SAG (20 mg/kg/d for 28 days). He did not return to the hospital for repeat bone marrow examination to document cure. Two weeks after completing treatment with SAG, he was readmitted with recurrence of fever for 1 week. On examination, he had severe pallor with splenomegaly (spine, 17 cm and liver, 4 cm below the costal margin). Investigations showed pancytopenia with hemoglobin of 4.9 g/dL, total leukocyte count of 2300/μL with 62% neutrophils, 70% lymphocytes, and platelet count of 97,000/μL. Bone marrow aspirate examination showed persistence of amastigote form of L. donovani. His LFTs and RFTs, serum electrolytes (sodium, potassium, and ionized calcium), and ECG during the previous admission before starting SAG were normal.

He received packed red blood cell transfusion, and amphotericin B was started as a slow intravenous infusion with a dose of 0.25 mg/kg/d and built up to 1 mg/kg/d in 4 days. The child was stable, accepting food, and was gradually improving, when completing 4 days after starting amphotericin B, he had a sudden cardiac arrest and died despite all aggressive measures to resuscitate him. The clinical deterioration was so sudden that an ECG could not be taken at that time. The sudden nature of the events indicates that the cause of death was a likely fatal cardiac arrhythmia.

CASE 3

A 7-year-old boy with kala-azar, presented for follow-up, was admitted for repeat bone marrow examination to document cure after completion of 28 days of intramuscular SAG. He was
asymptomatic at admission but had persistence of splenomegaly. Repeat bone marrow examination showed persistence of amastigote form of *L. donovani*. Treatment with amphotericin B was started on day 3 of admission after RFT, LFT, and electrolytes were confirmed to be normal. An ECG done previously before starting SAG was normal. Amphotericin B was started with a dose of 0.25 mg/kg/d and increased to 1 mg/kg/d and was administered as slow intravenous infusion lasting 4 hours. The child was stable, accepting food orally, and improving clinically until day 6 of admission. On day 6, he developed sudden deterioration and was found to be in shock. The child expired despite all supportive measures.

**DISCUSSION**

We have reported 3 cases of kala-azar in which administration of amphotericin B at an interval varying from 0 to 15 days after SAG therapy had stopped, resulted in sudden clinical deterioration and death of previously stable children. The first case had a documented ventricular tachycardia on day 2 of starting amphotericin B. In the other 2 cases, the clinical deterioration was unexpected and sudden, suggesting a fatal arrhythmia.

Conventional therapy for VL continues to be pentavalent antimony (SAG), despite its requirement of a long course, need for parenteral administration and increasing levels of resistance especially in some areas of Bihar. Serious life-threatening cardiotoxicity with the use of SAG is a potential threat. Electrocardiographic changes suggestive of cardiac toxicity include diminution in the height of the T wave, inversion of the T wave, elevation of the ST segment, prolonged QT interval, and diminution in the height of the P, R, and T waves. Cardiac arrhythmias (supraventricular arrhythmia / coarse atrial fibrillation, ventricular tachycardia, ventricular fibrillation, torsade de pointes, and multifocal ventricular ectopics) may also occur. Pharmacokinetics of SAG has 2 phases—an initial rapid elimination phase (1/2 of 2 hours) and a subsequent slow elimination phase (1/2 of 76 hours). The latter is thought to be responsible for cardiac toxicity and can persist for 2 months after cessation of therapy.

Amphotericin B-related cardiotoxicity is reported infrequently in the literature. In a single case it was due to inadvertent overdose of amphotericin B deoxycholate. In the other report, patient developed transient but reproducible hypoxemia, elevated pulmonary artery pressures, and depression of cardiac output during infusion of liposomal amphotericin B. In view of the increasing resistance to conventional drugs such as SAG in >60% cases for treatment of VL, it is important that cumulative toxicity of the 2 drugs, that is, amphotericin B and SAG be kept in mind before starting therapy for VL.

Cumulative cardiotoxicity of SAG and amphotericin B has been reported. In a study by Thakur et al, 3 of 7 cases of VL with myocardial damage secondary to SAG developed cardiac arrhythmias and cardiac arrest when amphotericin B was started without a rest period. If there was a rest period of 10 days after discontinuing SAG and if it was ensured that ECG changes had reverted before amphotericin B was started, then such events were not observed.

In another study by Jha et al, 34 patients with multidrug-resistant VL were treated with amphotericin B. In that series 1 patient died suddenly of myocarditis before the full dosage of drug was achieved. The ECG showed tachycardia with nonspecific ST and T-wave changes. The prior treatment details of the patient and time interval between drugs were not mentioned. Serum potassium and magnesium values were normal. Subsequent studies on amphotericin B for treatment of VL, when undertaken after taking due precautions, did not reveal any cardiac events.

The mechanism of this cardiotoxicity with coadministration of SAG and amphotericin B is not clear. The use of SAG possibly increases the susceptibility of the myocardium to damage by amphotericin B. It is prudent to take certain precautions before starting amphotericin B treatment in cases of VL who have previously received SAG. A rest period of approximately 14 days (or longer if ECG changes persist) and correction of any existing electrolyte abnormalities should precede treatment with amphotericin B.

**REFERENCES**


Molecular detection of the Australian grapevine yellows phytoplasma and comparison with grapevine yellows phytoplasmas from Italy

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Keywords: Australian grapevine yellows, phytoplasma, genetic relatedness, Australia, Italy

Abstract

A diagnostic test using the polymerase chain reaction is described for the detection of phytoplasma DNA in grapevines collected from South Australia and Victoria. Grapevines with Australian grapevine yellows disease tested positively for a phytoplasma but those with 'restricted spring growth syndrome' (formerly called 'grapevine decline') tested negatively. Restriction fragment length polymorphism analyses were done to determine the relationships between phytoplasmas of the Australian grapevine yellows and of representatives from both the aster yellows group (which includes phytoplasmas of grapevines from Italy) and the elm yellows group (which includes phytoplasmas of flavesence dorée). Results showed that Australian grapevine yellows is associated with a unique phytoplasma that is more closely related to the phytoplasmas of the aster yellows group than to those of the elm yellows group.

Abbreviations

The following abbreviations of disease types and molecular biology terms are used in this paper:
AGY Australian grapevine yellows; AY aster yellows; BN bois noir; EY elm yellows; FD flavesence dorée; FDB southern European grapevine yellows; FDU flavesence dorée strain, northern Italy; GY grapevine yellows; IPVR Italian pivertinkle virescence; MLO mycoplasma-like organism; RGSO restricted growth syndrome; SPL sweet potato little leaf; bp nucleotide base pair; PCR polymerase chain reaction; RFPL restriction fragment length polymorphism.

Introduction

Australian grapevine yellows (AGY) is a disease first reported in 1975 and first described by Magarey and Wachtel (1983, 1985, 1986b). Diseased grapevines exhibit yellowing, downward curling of leaves on stunted shoots that do not harden off but remain rubbery (Magarey and Wachtel 1985). Shoot tips cease growth and die, and bunches shrivel and fall (Magarey and Wachtel 1986a). In the main, cultivars producing grapes for premium white wines are affected; most damage was found in Chardonnay and Riesling, but symptoms were also seen on other cultivars, e.g., Traminer, Montils and Semillon. The disease is now present in nearly every viticultural district of Australia (Magarey and Wachtel 1986a). A survey in 1980 showed the highest incidence of disease occurring in the irrigation areas of the Riverland (from Waikerie to Renmark, South Australia). Sunraysia (centred on Mildura, Victoria) and of the Murrumbidgee (centred on Griffith, NSW), and in the Hunter Valley (NSW). Average crop losses of 10% were observed in vineyards of Riesling and Chardonnay. In some vineyards, 5% of all vines were diseased, and severely diseased vines showed yield reductions of 40-50% (Magarey and Wachtel 1986a).

Australian grapevine yellows is similar to other grapevine yellows diseases and to flavesence dorée. The latter is an important disease of grapevine in southwest France (Caudewell et al. 1971) and Italy (Belli et al. 1985) and is associated with a mycoplasma-like organism (MLO) (Caudewell 1980). The new trivial name for MLOs is phytoplasmas and this term is used here to refer to MLOs. Studies of injecting antibodies into vines diseased with Australian grapevine yellows indicated sensitivity to oxy-tetracycline hydrochloride but not to penicillin G, inferring a phytoplasma aetiology.
for the disease (Magarey and Wachtel 1986a). Also, the phloem sieve tubes showed considerably more fluorescence in diseased than in symptomless grapevines (Magarey and Wachtel 1985, 1986a, 1986b), and this is in accordance with observations of Namba et al. (1981). Subsequently, phytoplasma particles were observed in the phloem sieve tubes of diseased Australian grapevines (Magarey et al. 1988). These observations provided evidence that Australian grapevine yellows is associated with a phytoplasma. However, evidence is still required on the nature of the phytoplasma infection. As this disease is a persistent problem in Australia, a diagnostic test is needed for the screening of grapevines which would enable the production and distribution of planting material free of Australian grapevine yellows.

In the spring of 1993 and again in 1994, certain decline symptoms were first reported on Chardonnay and then on other cultivars in most of the major grapegrowing regions of south-eastern Australia. Observed symptoms of the condition, now named 'restricted spring growth syndrome' (RSGS, formerly called 'grapevine decline') included poor budburst and uneven, reduced shoot growth. Some vines even died (Mattschoss 1994). It is thought at present that a combination of physiological and environmental factors may stress vines which then exhibit growth problems, but contributions of secondary factors have not been ruled out. Phytoplasma infection may be one such factor and a diagnostic test for it would help to determine whether such an organism plays a role in RSGS.

Yellows disease of grapevines has been reported from many countries including France, Italy, Germany (Caudwell et al. 1971, Davis et al. 1992, Herschler 1937), USA (Pearson et al. 1985), Portugal (Parente et al. 1994) and Greece (Rumbos and Avgelis 1985). The genetic relatedness of phytoplasmas associated with this disease has recently received much attention and research has shown that a number of different phytoplasmas may be involved. The information gleaned is summarised here because it is important in relation to the present study. The type of grapevine yellows found in southern France and in northern Italy, called flavescence dorée (FD) was shown in recent molecular studies to be associated with a phytoplasma coded FDF in France (Daire et al. 1993) and FDV in northern Italy (Prince et al. 1993). FDV is confined to these two regions as shown by serology tests using FD-specific antibodies on samples of diseased vines from Sicily and Emilia-Romagna (Italy), Switzerland, Israel, USA, South Australia and South Africa (Kusza et al. 1993). In particular the phytoplasma of the CD found in southern France (i.e. FD) was different from that found in vineyards of northern France (Daire et al. 1993).

Similarly, Davis et al. (1993) provided evidence for two genomic clusters of grapevine phytoplasma in Italy, namely the FDV strain of flavescence dorée in the north and FDB, associated with the southern FDB phytoplasma, in the south. FDB phytoplasma was shown to be closely related to Italian periwinkle virescence phytoplasma (IPVR) by Davis et al. (1992) and both were distinct from FDV.

The phytoplasma FDB, together with the phytoplasmas of four GYs from Emilia Romagna vines (CA1, CH1, SAN1, SAN2), were shown to have the characteristics of phytoplasmas of the aster yellows (AY) group and were thought to represent a new subgroup within the AY group (Prince et al. 1993). In contrast, FDF proved in molecular studies to be related to the elm yellows (EY) phytoplasma group V (Daire et al. 1992, Prince et al. 1993, Smart et al. 1994, Daire et al. 1993, 1994, Kirkpatrick et al. 1994b, Seemüller et al. 1994) but not to be identical with the strain type of EY (Daire et al. 1994, Bertacini et al. 1995). The Italian FDV and the grapevine yellows phytoplasma from Virginia (USA) are members of the X-disease III phytoplasma group, according to evidence obtained by Prince et al. (1993), and similarly the grapevine yellows phytoplasma from New York was shown to be related to the western X-disease phytoplasma, which is in the X-disease group (Daire et al. 1994).

Bois noir is another important grapevine yellows disease which has the same symptoms as flavescence dorée but cannot be transmitted by the leafhopper vector of flavescence dorée (Caudwell et al. 1971). BN-diseased grapevines from southern and northern France, northern Italy, Sicily and Israel were shown to contain a stolbur-related phytoplasma (Daire et al. 1994). Results from sequence data analysis have placed the grapevine yellows from Germany called Vergilbungskrankheit and of BN from southern France within the stolburs (Smart et al. 1994, Kirkpatrick et al. 1994b, Seemüller et al. 1994). Parsimony analysis of 16S rRNA and 16S/23S spacer sequences has shown that the group 1 aster yellows can be divided into two subgroups, the aster yellows group and the stolbur group with Vergilbungskrankheit and bois noir from southern France (Seemüller et al. 1994, Smart et al. 1994). This suggests a close relationship between these 'stolbur-type' phytoplasmas and those currently associated with the aster yellows group.

In summary, RFLP and sequence data analysis on material from grapevines infected with grapevine yellows indicate an association of the disease with phytoplasmas belonging to four of the above groups: (1) FDF from France is characteristic of phytoplasmas from the elm yellows (EY) group V; (2) FDV from Italy and the grapevine yellows phytoplasmas from SA, Virginia and New York are characteristic of the X-disease group III; (3) FDB from the South and the four phytoplasmas of the grapevine yellows from Emilia Romagna of Italy (CA1, CH1, SAN and SAN 2) are characteristic of the aster yellows (AY) group I; and (4) bois noir (France, Italy, southern Italy and Israel) and Vergilbungskrankheit (Germany) are characteristic of the stolbur group.

Thus, much effort has gone into studying the genetic relatedness worldwide of the grapevine yellows diseases. Up to now, the only information about the relationship of the phytoplasma of the Australian grapevine yellows to other grape phytoplasmas comes from Kusza et al. (1993) whose serology test showed that this Australian phytoplasma is not closely related to FDF. While determination of the relationship between the phytoplasma of the Australian grapevine
yellows and of all other known phytoplasmas of
grapevine yellows may require lengthy studies, it
appeared important to initiate such work. It should
lead to a better knowledge of Australian grapevine yel-
lows, the origin of its phytoplasma and its distribution
in the Australian grapegrowing regions. The ability to
recognise phytoplasmas of grapevine yellows will also
be important for the safe movement of planting
material both nationally and internationally.

Materials and Methods

Source of phytoplasmas and extraction of nucleic acid from
plants
In Australia, samples from Chardonnay grapevines
with Australian grapevine yellows were collected in
South Australia (Loxton) and Victoria (Karadoc and
Robinvale). Samples from Chardonnay grapevines
with restricted spring growth syndrome were collected
at Walkerie (South Australia) and Karadoc, and from
asymptomatic Chardonnay and Riesling vines at
Loxton. Plants of sweet potato with sweet potato little
leaf phytoplasma (SPLP) came from Darwin (Northern
Territory), of petunia with petunia little leaf from
Canberra (Australian Capital Territory), of potato with
potato purple top wilt from Knoxfield (Victoria) and of
tomato with tomato big bud from Walkerie.

In Italy, samples of vines with symptoms of
grapevine yellows were collected from the cvs
Chardonnay, Trebbiano and Caveccia of the Emilia-
Romagna region, from cv. Prosecco in the Veneto
region and from cv. Alborola in Liguria, while samples
of asymptomatic vines came from cv. Chardonnay
vines of the Emilia-Romagna region. Additionally, host
plants of the respective species carrying disease were
obtained for Italian periwinkle virescence, Italian glad-
olus virescence and Italian ranunculus phylloclad. Total
nucleic acids were extracted in Australia from the
Australian samples using the methods of Gibb and
Padovan (1994) and Steenkamp et al. (1994), and in
Italy from the Italian samples using the method of Lee

Polymerase chain reaction (PCR)
The primer pair used in the initial screening of
Australian grapevines was 16R723f and m23SR. These
primers amplified a fragment of 1076 base pairs
extending from position 723 in the 16S rRNA gene
through the 16S/23S spacer region and into the start of
the 23S rRNA gene. The sequences of the forward
primer (16R723f) and the reverse primer (m23SR) are:
16R723f: 5'-GAAGGGCGCTGCTGGGTCT-3';
m23SR: 5'-TAGTGCCAAAGGCTACCTGTG-3'.

A second set of primers (P3/7P7) amplified approxi-
mately 300 base pairs of the 16S/23S spacer region
(sequence provided by C. Smart) and was useful for
screening Italian grape phytoplasmas from a range of
grapevine cultivars. A third set of primers (R16F2/R2)
which amplified a product of 1245 base pairs extend-
ing from position 152 to 1397 of the 16S rRNA gene
(Lee et al. 1993) was used to study genetic related-
ness by restriction fragment length polymorphism
(RFLP) analysis. A fourth set of primers (P1/P7) amplifi-
cy a larger region of approximately 1800 base pairs
of the 16S rRNA gene extending through the spacer
region and into the beginning of the 23S rRNA gene
(forward primer P1, Deng and Hiruki 1991) and
reverse primer sequence P7 (provided by C. Smart)
were also used in RFLP analysis as an adjunct to the
RFLP study of the smaller, 1245 base pair sequence.

For the PCR, nucleic acid extracted by the method
of Gibb and Padovan (1994) and Steenkamp et al.
(1994) was used without further dilution. The reac-
tions contained 0.2 mM of each dNTP, 0.4 μM of each
primer, DNA polymerase buffer supplied with the
enzyme 1 U Taq DNA polymerase (Boehringer Mann-
heim GmbH, Germany) and 1 μL of the nucleic acid
sample. The total reaction volume was 20 μL for gen-
eral screening in a Corbett FTS-1 thermocycler
(Corbett Research, Mortlake, NSW, Australia) or 50 μL
for PCR products to be used for RFLP analysis in a
Corbett FTS-320 thermocycler. Parameters for 40
cycles were denaturation at 94°C for 1 min, annealing
at 60°C for 30 s, and extension at 72°C for 30 s except
for the final cycle when extension was for 90 s. After
amplification, a one-tenth volume aliquot from each
sample was subjected to electrophoresis in a 1.2% agarose
gel and visualised by staining with ethidium bromide
and UV illumination. Nucleic acid extracted
after the method of Lee et al. (1991) was used in the
PCR at a concentration of 60 ng/μL for grapevine and
20 ng/μL for other plant host species. PCR conditions
for these samples were as described in Lee et al.
(1993). Total nucleic acid extracted from asymptomatic
plants were subjected to the PCR as control, and in
some experiments controls were included in which
the PCR mix contained water without plant nucleic
acid.

Restriction fragment length polymorphism analysis (RFLPA)
Ten μL of the PCR products amplified by using the
R16F2/R2 primers were digested separately using three restriction enzymes, Kpn I (10U), Alu I (6U)
(Fermentas, MBI, Vilnius, Lithuania) and Mse I (1U)
(Sigma-Aldrich, Milano, Italy) in buffers supplied by
the manufacturer. Digestions were incubated over-
night at 37°C and the fragments were visualised by
electrophoresis in a 5% polyacrylamide gel in 1×TBE
buffer followed by staining with ethidium bromide.
The gel was photographed using a UV transilluminator.
PCR products amplified by using the PI/P7 primers
were digested with Mse I (4U) (New England Biolabs,
Beverly, MA, USA) in buffers supplied by the manu-
facturer. For the PCR products of SPLP and AGY, 5 μL
of DNA were digested, whereas for the grapevine yel-
lows phytoplasma from Trebbiano, 15 μL were used
because the PCR product was in low concentrations
when visualised on an agarose gel. Digestions were
incubated overnight at 37°C and the fragments were
visualised by electrophoresis in 4% UltraClear Resolution
Agarose gel (Progen Industries, Darra, Queensland,
Australia) in 1×TBE buffer followed by staining with
ethidium bromide and photographed using a UV
transilluminator.
**Results**

Polymerase chain reaction

This work has shown that a PCR-based assay can be successfully used to detect phytoplasma DNA in grapevines with symptoms of Australian grapevine yellows. This test was first applied to amplify a PCR product of the 1076-bp region, using the primer pair 16S/23S (Figure 1). This assay was used with a range of host plant species. Nucleic acid extracted from asymptomatic plants did not exhibit any PCR product. Samples from grapevines infected with AGY and growing in Loxton, Karadoc and Robinvale gave PCR products of the same size as the positive controls from sweet potato and tomato (Figures 1 and 2). The extracts of grapevines with RSGS showed no PCR product (Figure 2).

Extracts from Italian grapevines of cvs Caveccia, Chardonnay, Prosecco and Trebbiano exhibiting symptoms of grapevine yellows disease, and samples from grapevines with Australian grapevine yellows, were screened using the primer pair P3/P7 which amplified the 16S/23S spacer region (approximately 300 base pairs). PCR products were observed for all Italian grapevine cultivars with AGY disease and for AGY (Figure 3). Some DNA samples from Italy gave faint bands despite repeated PCR tests; this DNA, when subjected to gel electrophoresis, was found to be partially degraded (data not shown) which may account for the faint PCR products. No PCR products were amplified in the asymptomatic control samples (Figure 3). There was some variation in the size of PCR products (Figure 3). While the P3/P7 primer pair proved to be useful for...
amplifying phytoplasma-specific DNA in samples that did not give a positive result with other primer pairs, the small size of the PCR product precluded its use in RFLP analysis. No bands were observed on samples from grapevines with 'restricted spring growth syndrome' (results not shown).

Restriction fragment length polymorphism analysis

To study genetic relatedness, a range of phytoplasmas was amplified in the PCR, using the R16F2/R2 primer pair, and compared by RFLP analysis. The phytoplasmas used in this study were from Chardonnay with AGY from Loxton, GY-diseased grapevines from Emilia-Romagna, Italian periwinkle virescence (IPVR), virescence of Italian gladiolus, phylloyd of Italian ranunculus and a GY-diseased grapevine from Liguria. These samples gave the expected 1245-base pair product (Figure 4) while PCR products were not observed in samples from asymptomatic grapevine, gladiolus, ranunculus, periwinkle or water controls. The 1245-base pair PCR products were subjected to RFLP analysis, using three restriction enzymes Alu I, Kpn I, and Mse I (Figure 5). The Australian grapevine yellows restriction pattern generated by digestion with Kpn I was the same as that for grapevine yellows from Emilia Romagna, IPVR, virescence of Italian gladiolus and for phylloyd of Italian ranunculus, but differed from that for grapevine yellows from Liguria (Figure 5). For the phytoplasma of Australian grapevine yellows, the restriction pattern generated by digestion with Alu I or Mse I was not the same as any of the Italian phytoplasmas tested (Figure 5).

The 16S rRNA gene and spacer region of Australian grapevine yellows and of an Italian grapevine yellows contained in Trebbiano was amplified by PCR using the P1/P7 primers (Figure 6). An Australian phytoplasma from sweet potato (SPLL) was also amplified in

Figure 6. PCR amplification of phytoplasmas from grapevines and from sweet potato. The primer pair P1/P7 was used; it amplifies almost the entire 16S rRNA gene and the spacer region between the 16S rRNA and 23S rRNA genes. M = DNA molecular weight markers; bp = base pairs; lane 1 = asymptomatic sweet potato; lane 2 = sweet potato with SPLL; lane 3 = asymptomatic Chardonnay (Loxton, SA); lane 4 = Chardonnay with AGY (Loxton, SA); lane 5 = asymptomatic Chardonnay (Emilia-Romagna); lane 6 = Trebbiano with GY (Emilia-Romagna)

Discussion

A molecular diagnostic test for phytoplasmas in grapevine based on amplification of a region of phytoplasma genome by means of the polymerase chain reaction was used to detect phytoplasma-specific DNA. Using this technique on samples from Chardonnay
vines showing symptoms of Australian grapevine yellows, we could demonstrate that phytoplasmas are associated with this disease. Phytoplasma-specific DNA was not present in detectable amounts in samples extracted from Chardonnay vines showing symptoms of the restricted spring growth syndrome.

Very little is known about Australian phytoplasmas and work so far has shown a wide distribution of a phytoplasma that is related to but not identical with the peanut witches' broom and Crotalaria witches' broom phytoplasmas. The type strain of this successful Australian phytoplasma is SPLL, the sweet potato little leaf phytoplasma (Gibb et al. 1995). Results reported in this paper show that the phytoplasma associated with Australian grapevine yellows is not the same as the SPLL phytoplasma and this provides the first evidence that there is more than one phytoplasma in Australia.

The finding that the size of the 16S rRNA gene and of the adjacent spacer region differ is not entirely surprising when one accepts that the various examined phytoplasmas are not the same. It is known that the nucleotide sequences flanking a conserved tRNAle in this spacer region vary considerably (Kirkpatrick et al. 1994a) and spacer sequences from different phytoplasmas vary considerably in length (Smart et al. 1994). In our case, the respective regions of the phytoplasma DNA were larger in the samples obtained from the Italian Trebbiano grapevine yellows than in those from the Chardonnay Australian grapevine yellows. This observation could be viewed as added evidence that the phytoplasma of the Australian grapevine yellows is not identical with that of the Italian grapevine yellows in Trebbiano. Other Italian grapevine samples were not used in this analysis because they could not be amplified using the P1/P7 primer pair.

The present study led us to examine the genetic relatedness of the AGY phytoplasma to phytoplasmas from AY group I and EY group V. As mentioned previously, these two groups contain grapevine phytoplasmas and the AY group includes a large number of phytoplasmas from a wide range of host plant species. RFLP analysis of the 16SrRNA gene has shown that some phytoplasmas placed in the AY group show polymorphisms with some restriction enzymes and this has lead to subgroupings of phytoplasmas within the group (Lee et al. 1993). The AY-related phytoplasmas used in this study belong to different subgroups within the AY group (Lee et al. 1993; Vibio et al. 1994), EY from Emilia-Romagna (group I-G) (Bertaccini et al. 1993a), IPVR (group I-C) (Davis et al. 1992), virescence of Italian gladiolus (group I-B) (Bertaccini et al. 1993b) and phyllyd of Italian ranunculus (group I-C) (Bertaccini et al. 1994). The EY-related phytoplasma used in this study was GY from Liguria (group V) (Bertaccini et al. in press).

AGY was shown by RFLP-analysis to differ from the GY sample for Liguria whose phytoplasma is classified, together with flavescence dorée, as belonging to the EY group. Therefore, AGY ought to be different from flavescence dorée also, a result which supports previous findings that FD-specific antibodies did not detect AGY (Kuszaa et al. 1993). In contrast, the phytoplasma of AGY gave restriction fragment patterns after digestion with Kpn I that were identical with those of phytoplasmas of the AY group. However the restriction fragment patterns of AGY phytoplasma were different from all other known phytoplasmas, including phytoplasmas related to the AY group when digestion was done with either Alu I or Mse I. Thus, based on the restriction fragment pattern, the AGY phytoplasma was closest to, but not identical with, the CY phytoplasma from the Emilia-Romagna and the IPVR phytoplasma (AY group I-G).

Conclusions
This work has reconfirmed that molecular diagnostic tools using the polymerase chain reaction to amplify specific sequences can be used to detect grapevine phytoplasmas. Grapevines with Australian grapevine yellows tested positive for phytoplasmas while those with restricted spring growth syndrome did not. From this first study it appears that the phytoplasma associated with Australian grapevine yellows is not identical with any known phytoplasma, but that it is most closely related to phytoplasmas from the aster yellows group which includes the phytoplasms of southern European grapevine yellows and of four phytoplasmas from Emilia-Romagna vines. Further study is required to determine the relationship between grape phytoplasmas currently in the aster yellows group and those in the stolbur group because some workers have used sequence data to show a close relationship between these two groups. Evidence presented here shows the phytoplasma of Australian grapevine yellows not to be related to a grape phytoplasma from the elm yellows group V. Flavescence dorée is a member of this group, our results therefore indicate that Australian grapevine yellows is not related to flavescence dorée. The phytoplasma of Australian grapevine yellows appears to have arisen either de novo in Australia or originated from the aster yellows group of phytoplasmas of European grapevine yellows, but evolved in Australia as shown by its unique restriction enzyme pattern.

Acknowledgements
For financial support, Ms Padovan and Dr Gibb thank the Australian Research Council and the Northern Territory University Internal grants scheme, Dr Bertaccini and Ms Vibio thank the Ministero delle Risorse Agricole, Alimentari e Forestali, Rome, Italy for the special grant 'Grapevine Flavescence Dorée' and Mr Bonfiglioli thanks Southcorp Wines Pty Ltd.

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Manuscript received: 13 March 1995