In developing countries, viruses causing respiratory disease are a major concern of public health. During January 2010–December 2011, 2,737 patients with acute respiratory infection from the outpatient departments as well as patients admitted to hospitals were screened for different respiratory viruses. Nasal and or throat swabs were collected and transported to the laboratory where initial screening of influenza A and influenza B viruses was performed. The samples were tested further for influenza C virus, parainfluenza viruses 1–4, human rhinovirus, metapneumovirus and respiratory syncytial virus by conventional RT-PCR. The study revealed that the majority of the patients were under 5 years of age; both due to their higher susceptibility to respiratory infections and presentation to hospitals. Out of 2,737 patients enrolled in this study, 59% were found positive for one or more respiratory viruses. Influenza B infection was detected in 12% of patients followed by influenza A (11.7%), respiratory syncytial virus (7.1%), parainfluenza virus-2 (6%), metapneumovirus (3%), parainfluenza virus-3 (1%), parainfluenza virus-4 (0.6%), parainfluenza virus-1 (0.3%), influenza C (0.2%) and human rhinovirus (0.2%). Distinct seasonal infection was observed only for influenza A and influenza B viruses. J. Med. Virol. 85:1459–1465, 2013. © 2013 Wiley Periodicals, Inc.

KEY WORDS: influenza like illness; respiratory viruses; Surveillance in eastern India; parainfluenza virus; metapneumovirus
5–10% of acute respiratory infections only in children [Chen et al., 2004]. In addition, infections caused by other respiratory viruses such as parainfluenza viruses and coronaviruses occur worldwide. Furthermore, coronavirus NL63 [van der Hoek et al., 2004], coronavirus HKU [Fouchier et al., 2004; Woo et al., 2005] and bocavirus [Allander et al., 2007] are also responsible for acute respiratory infections in pediatric, elderly and immunosuppressed patients. Most studies based on laboratory diagnosis in hospitals are still restricted to influenza and respiratory syncytial virus. On the other hand, emerging respiratory viruses have been a subject of concern because of the risk of rapid spread and high fatality rates due to lack of both diagnosis and effective antiviral therapy.

In developing countries where diagnosis of viruses causing respiratory disease is restricted to a few laboratories, the etiology is undefined in a significant proportion (>60%); which undermines the impact of acute respiratory infections on health and economic burden [Monto, 1994; Henrickson et al., 2004]. In eastern India, frequency and genetic diversity of influenza viruses during 2005–2009 have been reported [Agrawal et al., 2010], but no information is available from the Indian subcontinent regarding other respiratory viruses. The present study aimed to identify common circulating respiratory viruses in addition to influenza during January 2010 through December 2011 in the eastern region of India.

MATERIALS AND METHODS

Sample Collection and Transportation

Nasal and/or throat swabs were collected from patients with influenza like illness attending the outpatient departments of hospitals in Kolkata, West Bengal, as described previously [Agrawal et al., 2009a]. In addition, swabs from patients with severe respiratory illness admitted to different hospitals in West Bengal were also referred to the laboratory for diagnosis. [Mukherjee et al., 2010]. Informed consent forms and detailed case histories were recorded before the collection of a specimen. The study was approved by the Institutional Ethical Committees.

RNA Extraction

Viral RNA was extracted by using commercially available QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) as per manufacturer’s instructions.

Screening for Influenza A/B Viruses by Real-Time PCR

For initial detection of influenza viruses, amplification of the matrix (M) gene of influenza A and influenza B viruses were carried out by real-time RT-PCR as described previously [Agrawal et al., 2009a].

Screening for Other Respiratory Viruses by Conventional RT-PCR

Respiratory viruses other than influenza were detected using a multiplex RT-PCR technique. First-strand cDNA synthesis was achieved using random primers and reverse transcription system (Invitrogen, Carlsbad, CA). The resulting cDNA was then subjected to multiplex PCR to enable simultaneous detection of multiple viruses. Primer sequences used in the study are described in Table I. The amplified products were then separated by agarose gel electrophoresis and visualized under UV after ethidium bromide staining. All samples were tested independently three times, using negative and positive controls to exclude cross-contamination of samples.

Statistical Analysis

Distribution frequencies of respiratory viruses were compared using Pearson’s chi square test and Fisher’s exact test. Continuous variables for population parameters such as age, laboratory investigations and other parameters were compared using one-way analysis of variance.

RESULTS

Demographic Characteristics of Outpatients

From January 2010 through December 2011, 2,737 swab samples from patients with respiratory infections were obtained; 1,574 (57.5%) of the patients were

| Strain                  | Primer name | Sequence (5’-3’) | Position | Product size (bp) |
|-------------------------|-------------|------------------|----------|------------------|
| Parainfluenza virus-1   | HPIV-1 (F)  | (+)CCG GTA ATT TCT CAT ACC TAT G  | 2–21     | 317              |
|                         | HPIV-1 (R)  | (−)CCCT TGG AGC GGA GTT GTT AAG | 298–318  |                  |
| Parainfluenza virus-2   | HPIV-2 (F)  | (+)AAC AAT CTG CTG CAG CAT TT   | 713–732  | 489              |
|                         | HPIV-2 (R)  | (−)ATG TCA GAC AAT GGG CAA AT   | 1,182–1,201 |               |
| Parainfluenza virus-3   | HPIV-3 (F)  | (+)CTG GAG GTT GTC AGG AGG TAG  | 632–652  | 195              |
|                         | HPIV-3 (R)  | (−)CTT TGG GAG TTG AAC ACA GTG  | 806–826  |                  |
| Parainfluenza virus-4   | HPIV-4 (F)  | (+)CTG AAC GGT TGC ATT CAG GT   | 1,969–1,988 | 442         |
|                         | HPIV-4 (R)  | (−)CTG CAT CAA GAA TGA GTC CT   | 2,391–2,410 |              |
| Influenza C             | Inf-C (F)   | (+)ACA CTT CCA ACC CAA TTT GG   | 676–695  | 485              |
|                         | Inf-C (R)   | (−)CCT GAC ACG AAT CAC CCT AT   | 1,141–1,160 |             |
| Human rhinovirus        | HRV (F)     | (+)GGG ACC AAC TAC TTT GGG TGT CCG TG | 304–323 | 113              |
|                         | HRV (R)     | (−)GC A TCT GGY ABY TCC CAC CAC CAN CC | 397–416 |                  |
males and 1,163 (42.5%) were females (ratio M/F = 1.35). Out of these 2,737 patients 49.4% were obtained from patients under the age of 5 years old. Remaining 50.6% patients were of 5–15 years (14.2%), 15–35 years (19.4%), 35–45 years (6.1%), 45–60 years (7.3%) and >60 years (3.7%), respectively.

Prevalence of Respiratory Viruses

Out of 2,737 samples, 1,616 (59%) were positive for one or more respiratory viruses; of which 11.7% were positive for influenza A; which comprised both influenza A(H1N1)pdm09 (6.7%) and influenza A(H3N2) (5%), influenza B was detected in 12% cases, influenza C (0.2%), parainfluenza virus-1 (0.3%), parainfluenza virus-2 (6%), parainfluenza virus-3 (1.0%), and parainfluenza virus-4 (0.6%), respiratory syncytial virus (7.1%), metapneumovirus (3.0%) and human rhinovirus were found in 0.2% cases (Fig. 1).

Co-Infection of Respiratory Viruses

During the study period, 64 samples were found to be co-infected with respiratory syncytial virus and influenza B viruses. Metapneumovirus was found together with respiratory syncytial virus (n = 16), influenza A (n = 1), and influenza B (n = 3) viruses. Mixed infection with influenza A and influenza B was detected in 13 cases.

Age Distribution

The prevalence of viral infection in different age groups has been shown in Figure 2. Infections due to influenza A(H3N2), influenza B, metapneumovirus and respiratory syncytial virus was high in children under 5 years of age followed by 5–15 years and 15–35 years; whereas, adults (15–35 years) and the elderly people were infected mainly with influenza A(H1N1)pdm09 virus. Influenza C virus infection predominantly occurred in children under the age of 5 years; although the frequency is very low. Similarly higher percentage of parainfluenza virus-1 infection was observed among children (under 5 years of age) followed by 15–35 years of age. In other age groups parainfluenza virus-1 was not observed in this study. Elderly persons (≥60 years of age) were at high risk for infection with parainfluenza virus-2 and -4, followed by the patients of 45–60 and patients under 5 years of age; whereas the infection rate for parainfluenza virus-3 was high among the patients under
5 years of age. For human rhinovirus infection, the risk group was 35–45 years followed by 45–60 years. During this study, all mixed infections were observed only in children aged less than 5 years.

**Seasonal Distribution**

The correlation of meteorological variations with the prevalence of respiratory viruses in eastern India is shown in Figure 3. Due to the pandemic in 2009, only influenza A(H1N1)pdm09 strain was found in 2010, however influenza A(H3N2) emerged again in 2011. Influenza B was found mainly after the monsoon season (October–December). Seasonal fluctuations for influenza C virus, parainfluenza virus 1–4, metapneumovirus and respiratory syncytial virus infection have not been observed during this study.

**DISCUSSION**

In 2004, surveillance for influenza viruses was expanded to India, as part of the global influenza surveillance network. However specific diagnosis which requires laboratory tests, were not widely available in eastern India. Hence the information on epidemiology and clinical features of respiratory virus infection in India is based entirely on research studies and the disease burden or seasonal prevalence of respiratory viruses remains largely undefined. This study initiated to complete the information on circulating respiratory viruses among patients attending the outpatients departments of different hospitals with acute respiratory infections in the eastern region of India during 2010 through 2011.

![Figure 3](https://example.com/figure3.png)

**Fig. 3.** Correlation of meteorological variations with prevalence of (A) influenza A, influenza B, influenza C, respiratory syncytial virus, metapneumovirus and (B) parainfluenza virus [1–4] during 2010–2011 in eastern India.

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During this surveillance, influenza B infection was found to be most prevalent followed by influenza A. In 2010, only influenza A(H1N1)pdm09 virus circulated whereas in 2011, only influenza A/H3N2 was found. This was in contrast with the previous report from eastern India, where higher prevalence of influenza A was observed compared to influenza B [Agrawal et al., 2010]. Increased frequency of influenza B infection could be due to the post-pandemic effect of influenza A(H1N1)pdm09 following which seasonal influenza A/H1N1 and influenza A/H3N2 viruses disappeared during 2009–2010. This is consistent with studies from Vietnam [Do et al., 2011] and other Asian countries [Mathisen et al., 2010]. Metapneumovirus has been associated with both upper and lower respiratory tract infections in children (3–13%) [van den Hoogen et al., 2001; Bosis et al., 2005; Gerna et al., 2005; Choi et al., 2006; Sarasini et al., 2006; Boivin et al., 2007]. During this study period, metapneumovirus was found in only 3% samples. This finding corroborates with reports from Canada, England and the USA, where the infection rate due to metapneumovirus varies from 2% to 4.5% [Stockton et al., 2002; Falsey et al., 2003; Boivin et al., 2004], but is significantly less compared to the prevalence in Netherlands (10%), Australia (9.7%), and Chile (5.4%) [van den Hoogen et al., 2001; Luchsinger et al., 2005; Mackay et al., 2006]. Respiratory syncytial virus which is the most common etiological agent of viral lower respiratory tract infections in infants and young children in the world caused 33.8 million new episodes in children <5 years of age [Nair et al., 2010] and is also associated with hospital admission of 3–9 per 1,000 children below 1 year [Pancer et al., 2011]. In Thailand alone, 417.1/100,000 incidences of pneumonia per year are attributed to respiratory syncytial virus [Olsen et al., 2010]. Worldwide, respiratory syncytial virus has been identified as the cause of 3.4 million cases of acute lower respiratory infections requiring hospital admission. Among all age groups, 7.1% cases were found positive for respiratory syncytial virus during 2010–2011 in this study. Following respiratory syncytial virus, parainfluenza virus-2 was the most predominant virus (6%) compared to parainfluenza virus-1, -3 or -4.

Consistent with surveillance results during 2006–2009, in eastern India [Agrawal et al., 2010; Roy et al., 2011] and in Bangladesh [Zaman et al., 2009] children under 5 years old were found to be most vulnerable to infections due to influenza B and influenza A (H3N2) viruses. Whereas, influenza A (H1N1)pdm09 affected adults and the elderly which is consistent with findings from other studies [Cauchemez et al., 2009; John and Moorthy, 2010; Mukherjee et al., 2010]. Similar to influenza viruses, metapneumovirus infection was also detected mainly in children (under 5 year of age), suggesting early acquisition of infection [van den Hoogen et al., 2001; Lu et al., 2011; Zappa et al., 2011]. During 2007–2008, only 8.7% of respiratory syncytial virus infection was found among children [Agrawal et al., 2009b]; whereas in the same geographical region, 13.7% samples were found to be positive for respiratory syncytial virus in children (under 5 years) during this study period (2010–2011). The higher prevalence of respiratory syncytial virus during this study period could be due to lower activity of influenza A viruses compared to the activity of influenza A viruses in 2007–2008 [Agrawal et al., 2009b]. Among children with acute respiratory infections, co-infection with one or more respiratory viruses has been observed [Bonzel et al., 2008; Canducci et al., 2008] which correlates with results of present study, where predominant mixed infections were observed only in children.

Unlike temperate countries where the prevalence of seasonal influenza may reach epidemic proportions during the winter months [John and Moorthy, 2010], in tropical countries like India, year round circulation of strains has been reported, though the infection peaks during rainy season (June–September) [Agrawal et al., 2009a, 2010]. There could be several factors such as socio-economic, environmental, education, overcrowding and other factors, which could affect the seasonal incidence and distribution of viral infections. In eastern India, influenza B was found to be prevalent after the monsoon season as well as in the winter months [Roy et al., 2011], and influenza A viruses predominated during the monsoon (June–July) [Agrawal et al., 2009a; Mukherjee et al., 2010]. During 2010–2011 seasonal infection due to influenza A [influenza A(H1N1)pdm09 or influenza A/H3N2] correlated with previous reports. However the seasonal pattern of infection with respiratory syncytial virus during 2010–2011 was found to be different from the previous reports from Kolkata, India [Agrawal et al., 2009a,b] and Bangladesh [Huq et al., 1990], where respiratory syncytial virus infection was found more commonly in the winter months or the dry seasons. Such variations in seasonal incidence are difficult to explain, as virus infection could be affected by a large number of different factors [Weber et al., 1998]. Previous studies in temperate climates showed that parainfluenza virus-1 and -2 infection occurs annually [Laurichesse et al., 1999; Karron and Collins, 2007], parainfluenza virus-4 infection occurs twice-yearly during the late fall and winter [Aguilar et al., 2000; Vachon et al., 2006; Lau et al., 2009] and parainfluenza virus-3 infection occurs mainly during late spring and summer [Laurichesse et al., 1999]. These differences may be attributed to different geographical regions and study years. However, as the numbers of positive cases were very low in the present study, the seasonal prevalence of influenza C, parainfluenza viruses and human rhinovirus could not be determined. Hence large-scale studies over a broader geographical range and longer time period are required to understand the seasonal infection pattern of respiratory viruses in India.

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