Development of Rapid Diagnostic Reagents for Respiratory Tract Infections in Children

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ABSTRACT

Rapid diagnosis of respiratory tract infections is important not only from the standpoint of clinical diagnosis but also infection control. The purpose of the present study is to investigate the possibility of diagnosing with the immuno-chromatography for pathogens of respiratory tract. A prospective, population-based study covering the total population less than 15 years of age in Tochigi, Japan, during the period of December 2010 to March 2013. Nasopharyngeal swabs were collected from 200 children with respiratory tract infections. RT-PCR analysis was used as the reference assay. The correlation of the results between obtained by the diagnostic reagents and RT-PCR assays was investigated. Pathogens were identified in a total of 64 (32.0%) out of 200 patients by RT-PCR as follows: respiratory syncytial (RS) virus, 29; M. pneumoniae, 17; metapneumovirus, 7; adenovirus, 5; influenza A virus, 3; and influenza B virus, 3. More than 100 copies/µl of Mycoplasma pneumoniae was obtained. Minimum detection sensitivity of the immuno-chromatography in the clinical material was the 4×10^1 copies/micro-liter. The correlation of the results between obtained by the diagnostic reagent for measles and RT-PCR was the positive match rate of 45.5% and negative match rate is 100%. From 79.0 to 100% of RT-PCR positive samples more than 4×10^1 copies/micro-liter was detected. The importance of M. pneumoniae and RS virus in the etiology of respiratory tract infections in was confirmed. Immuno-chromatography can be applied as a simple rapid diagnostic method from the beginning of the disease in general clinical practice.

Key Words: *Rapid diagnosis, immuno-chromatography, RT-PCR, respiratory tract infection, virus.*

Çocuklarda Solunum Yolu Enfeksiyonları İçin Hızlı Tanı Reaktiflerinin Gelişimi

ÖZET

Solunum yolu enfeksiyonlarının hızlı tanısı sadece klinik tanı açısından değil, aynı zamanda enfeksiyon kontrolü açısındanda önemlidir. Bu çalışmanın amacı, solunum yolu patojenleri için imünokromagrofi ile teşhis olasılığını araştırmaktır. Mart 2013 ile Aralık 2010 dönemindeki Japonya'nın Tochigi bölgesindeki yaşları 15'den daha küçük olan, toplam nüfusu kapsayan toplum temelli, prospektif

bir çalışmadır. Solunum yolu enfeksiyonu olan 200 çocuktan nazofarenks sürüntü alındı. Referans yöntem olarak RT-PCR analizi kullanıldı. Tanı reaktifleri ve RT-PCR ölçümleri ile elde edilen sonuçlar arasındaki korelasyonu araştırıldı. Patojenler, RT-PCR ile 200 hastanın toplam 64 (%32.0)' ünde aşağıdaki gibi tespit edilmiştir: Solunum sinsisyal (RS) virüsü, 29; M. pneumoniae 17; metapnömovirüsü, 7; adenovirüs, 5; influenza A virüs, 3; ve influenza B virüsü, 3. Mycoplasma pneumoniae 100 kopya/µl'den daha fazla elde edildi. Klinik materyalde imünokromagrafinin minimum tanısal duyarlılığı 4×10^1 kopya/mikrolitre idi. Kızamık için tanı ayıracı ve RT-PCR ile elde edilen sonuçlar arasındaki korelasyonun pozitif eşleşme oranı % 45,5 ve negatif eşleme oranı% 100 idi. RT-PCR'ın % 79.0 – 100'ünde pozitif örnekler 4×10^1 kopya / mikrolitreden daha fazla tespit edildi. Solunum yolu enfeksiyonlarının etiyolojisinde, M. pneumoniae ve RS virüsü önemi teyit edilmiştir. İmmüno kromatografi genel klinik uygulamada hastalık başlangıcından itibaren basit, hızlı bir tanı yöntemi olarak uygulanabilir.

Anahtar Kelimeler: *Hızlı tanı; immuno-kromatografi; RT-PCR; solunum yolu enfeksiyonu; virüs.*

INTRODUCTION

Establishment of rapid diagnosis of respiratory viral and mycoplasmal infections in children is important not only from the standpoint of clinical diagnosis but also infection control (1,2). Since atypical cases of pediatric respiratory infections are not uncommon, diagnosis based on laboratory testing is required (3,4). Laboratory diagnosis by serum IgM antibody-detection, reverse Transcription (RT)-PCR assay, and viral isolation are now established for the confirmation of the diagnosis of respiratory infections. Because of high sensitivity and specificity, these methods are the most reliable for laboratory diagnosis of viral and mycoplasmal infections (5,6). However, although they are effective for epidemiological investigation, they are not suitable for use in daily clinical practice (7). Not from the importance of rapid diagnosis, the experimental laboratory diagnosis of respiratory infections has been reemphasized to establish the daily application in general clinics.

There are several different assays to get the diagnosis of respiratory tract infections such as influenza. The gold standard for define diagnosis of influenza is virus isolation with tissue culture which usually takes 2 to14 days after inoculation of clinical specimens. Detection of virus-infected cells in nasopharyngeal secretions by direct or indirect immunofluorescent staining is widely used but needs techniques of collection samples. It still requires at least 2 hours getting the results. RT-PCR remains the method of choice for clinical diagnosis of special influenza viruses in respiratory specimens and for differentiating it from seasonal viruses (1).

Differentiation of influenza virus from other respiratory viruses is of prime importance because influenza is associated with higher rates of morbidity and mortality, is potentially preventable by vaccination, and can now be treated with specific antiviral agents (8). Since the diagnosis of influenza can be difficult when based solely on clinical symptoms, rapid diagnosis of influenza permits the initiation of antiviral therapy within a beneficial time frame, can result in discontinuation of inappropriate antibiotics, and prompts infection control measures to reduce spread in healthcare settings (9).

The immuno-chromatographic method, including lateral flow-based rapid diagnosis reagents, is widely used for daily point of-care testing in almost all Japanese clinics (10). These reagents have been developed and marketed for the diagnosis of respiratory infectious disease such as influenza. The rapid influenza diagnostic tests based on immunochromatographic lateral flow-based rapid diagnosis reagents flow, is currently the best choice for screening samples for the diagnosis of influenza virus due to its rapid detection ability, simple operation and low cost. This method is unable to further classify influenza A virus subtypes and is not as sensitive as RT-PCR. It can provide a rapid diagnosis of influenza A or B infection to aid clinical management in half an hour.

We already reported about the fundamental results of rapid diagnostic reagent for measles (11,12). To detect the measles virus in clinical samples, a reagent has to react with each of wild 23 genotypes (13). In a previous study, more than 100TCID50 of measles virus was detected from nasopharyngeal swabs (11,12). The purpose of the present study is to investigate the possibility of diagnosing with rapid diagnostic reagents for viral and mycoplasmal antigens of pediatric respiratory tracts.

MATERIAL AND METHOD

Clinical specimens

We conducted a prospective, population-based study covering the total population less than 15 years of age in Nasu-shiobara, Tochigi, Japan, during the period of December 2010 to March 2013 (Figure 1, Figure 2).

During the time of April to June 2011 the investigations were interrupted due to the big earthquake which attacked east part of Japan including Tochigi Prefecture. Nasopharyngeal swabs were collected from 200 children with respiratory-tract infections. Results are expressed as the equivalent of copies per micro-liter according to titration of control RNA. The correlation of the results w as investigated.

Specimen collection was performed by the authors' responsibilities under the agreement based on ethical guidelines on clinical research (issued by the Ministry of Education and Science and the Ministry of Health, Welfare and Labor of Japan) and the Helsinki Declaration (World Medical Association). All study procedures were approved by the Research Ethics Committee of International University of Health and Welfare, Tochigi, Japan (Reference No. YM 10-08).

The lateral flow-based immuno-chromatography

In order to select the target protein for the lateral flow-based rapid diagnostic kit, 25 reagents were prepared with previously-developed antibodies for measles H, F, M and N proteins, and the detection limits of them were examined using cultured virus of Edmonston strain (14,15). B4 antibody to N protein exhibited 10 times higher sensitivity than the other antibodies (16). Rated measurement sensitivity using cultured measles virus, about reagent system already includes a wild stock.
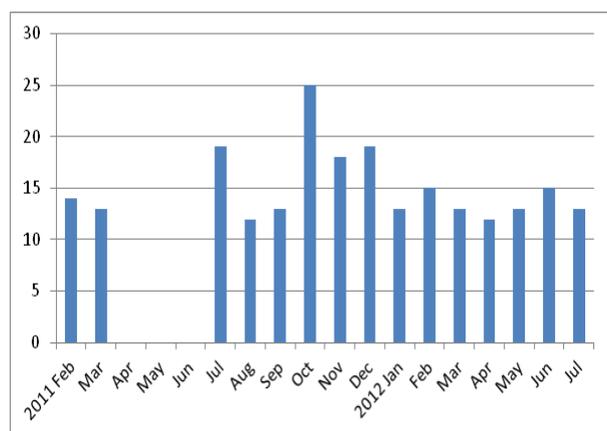
Evaluation of the diagnostic reagent using clinical samples

During the time of March 2008 to July 2010 clinical samples were obtained from the 46 patients (0 to 38 years of age; average 8.1 years, 25 males, 21 female) with suspected measles infection at 14 medical facilities in the area of Tokyo, Chiba, Osaka and Hokkaido of Japan. In the collaboration facilities, nasopharyngeal specimens of patients were collected and diagnosed with the test kit. Patients suspected measles clinical findings more than throat swab taken, evaluation of rapid tests kits. The residual samples for the diagnostic reagent were analyzed with RT-PCR in the laboratory.

We examined the correlation between the diagnostic reagent and RT-PCR (17,18), and the clinical symptoms in non-measles patients. Detection sensitivity compared with RT-PCR method using the clinical material collected from this time at 14 facilities.

Specimen collection was done by the physicians' responsibilities under the agreement based on ethical guidelines on clinical research (issued by the Ministry of Education and Science and the Ministry of Health, Welfare and Labor of Japan) and the Helsinki Declaration (World Medical Association).

Quantitative real-time RT-PCR assay

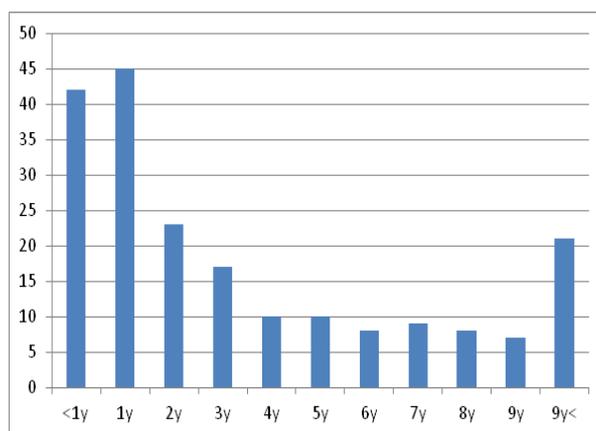Real-time RT-PCR analysis was carried out using primers and protocols previously described (17,18). For the Taq

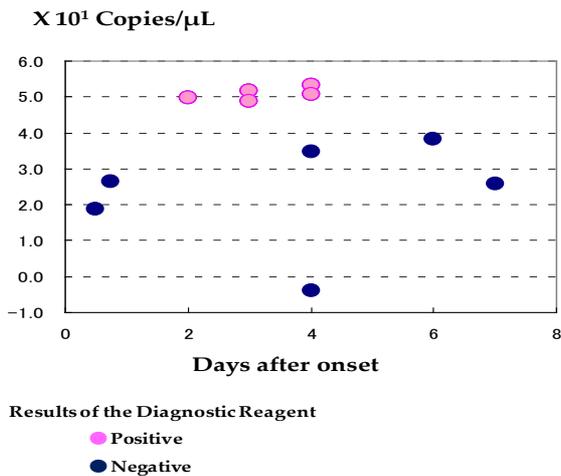***Figure 1**. Monthly Distribution of Patients*
(Monthly Cases, n=200; April to June 2011 were interrupted by earthquake).

***Figure 2**. Age Distribution of Patients (n=200).*

X 10¹ Copies/μL

Figure 3. *Time Distribution of Positive Specimens by RT-PCR assay.*

Figure 4. *Number of Copies in Clinical Specimens more than 40 copies/micro-liter, Detection Limit of Immunochromatography by RT-PCR.*

Man PCR method, we used the N3 primer and probe set for measles described in a previous paper (12). One-step RT-PCR reactions were performed in duplicate in an eight well optical micro tube (Applied Biosystems, Carlsbad, CA). A One Step Prime Script RT-PCR Kit (Takara-Bio, Otsu, Japan) was used for the PCR reagent. Briefly, a 25 μl reaction was set up containing 5 μl of template RNA, 12.5 μl of 2 X RT-PCR master mix, 1 μl of 25 X RT-PCR enzyme mix, 0.5 μl of Probe (20 μM), 0.5 μl of each of the primers (40 μM) and 5 μl of RNase-free water. A 5 mL RNA sample or control RNA sample was analyzed in 25 mL of reaction mixture. Thermal cycling conditions consisted of 45˚C for 10 min, followed by 95˚C for 10 min and then 40 cycles of 95˚C for 15 s, 60˚C for 45 s. Real-time assays were performed with the ABI PRISM 7500 sequence detection system (Applied Biosystems). Threshold cycle (Ct) values were calculated for each reaction. Samples were considered positive only if Ct values were less than 40 for both replicates. Results are expressed as the equivalent of copies mL, according to titration of control RNA. Viral RNA and non-viral RNA were used as positive and negative controls, respectively while RNase free water was used as blank control.

Nucleic acid extraction, primer design, and PCR conditions for other viruses and Mycoplasma were carried out as described previously (19,20,21). Duplicate aliquots of each specimen underwent automated NucliSens total nucleic acid extraction according to the manufacturer's instruc-
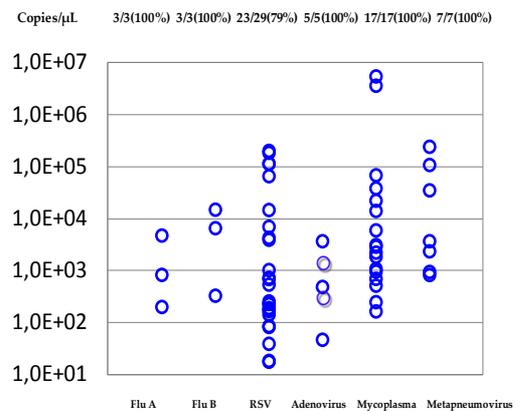
tions (22,23,24). RNA extracts either were immediately tested by RT-PCR or were stored at –70˚C in nuclease-free water containing RNase inhibitor. Oligonucleotide primers for respiratory syncytial (RS) virus, *M. pneumoniae*, metapneumovirus, adenovirus, influenza A virus and influenza B virus were designed to achieve standardized annealing temperatures (20,22). Primers for human β-actin mRNA were used to test for adequacy of the specimen, extraction, and RT-PCR procedure. The positive-strand primer of each set was labeled at the 5′ end with the fluorescent dye 6-carboxyfluorescein to enable automated GeneScan software analysis (Applied Biosystems). RT-PCR assays for each virus were also individually performed.

RESULTS

Sensitivity and specificity of the lateral flow-based immuno-chromatography diagnostic reagent system

In 46 clinical specimens for measles 5 were positive by the diagnostic reagent and 11 were positive by RT-PCR assay. These 5 reagent-positive specimens were also positive by RT-PCR assay (Figure 3). Minimum detection sensitivity of the reagent system in the clinical material was the 1 x 104 copies/micro-liter. Specimens were positive in onset later in 2-4 clinical days of measles, 7 samples in the RT-PCR assay. The correlation of the results between
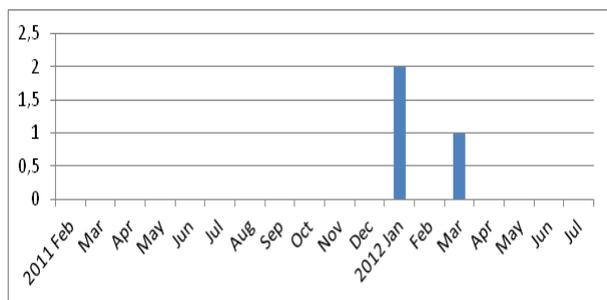
Figure 5a Cases of Influenza A (n=3).

Figure 5a-f. Time distribution of Etiologic Agents by RT-PCR assay.

Figure 5b Cases of RS virus (n=29).

Figure 5a-f. Time distribution of Etiologic Agents by RT-PCR assay.

obtained by the diagnostic reagent for measles and RT-PCR was the positive results that match rate of 45.5%. Negative in the entire RT-PCR negative example of 35 samples was a negative match rate is 85.4%. From 79.0 to 100% of RT-PCR positive samples of other viruses and *Mycoplasma* copies more than 4 x 10^{1}copies/micro-liter of positive results were obtained (Figure 4). More than 4 x 10^{1}copies/micro-liter were detected from 3/3(100%) of Influenza A, 3/3(100%) of Influenza B, 23/29(79%) of RS virus, 5/5(100%) of Adenovirus, 17/17(100%) of *M. pneumoniae* and 7/7(100%) of Metapneumovirus samples positive by RT-PCR assay. We detected 100 copies in the sample/μl or more antigens of *M. pneumoniae* and meta-pneumovirus which were difficult for detection by the conventional method.

Time distribution of positive specimens by the lateral flow-based immuno-chromatography diagnostic reagent system and by RT-PCR assay

We also examined the correlation between the diagnostic reagent and RT-PCR assay, and the symptoms in non-measles patients. In what had been diagnosed with measles in clinical findings so far Koplik's spots, judged measles diagnosis differential diagnosis difficult clinical symptoms alone was RT-PCR negative results. Although non-measles patients showed measles-like symptoms, the diagnostic reagent did not exhibit false-positive. We confirmed that the diagnostic reagent could detect measles in clinical samples. The diagnostic reagent detected 71% (5/7) of measles cases at 2-4 day after onset of fever (Figure 3).
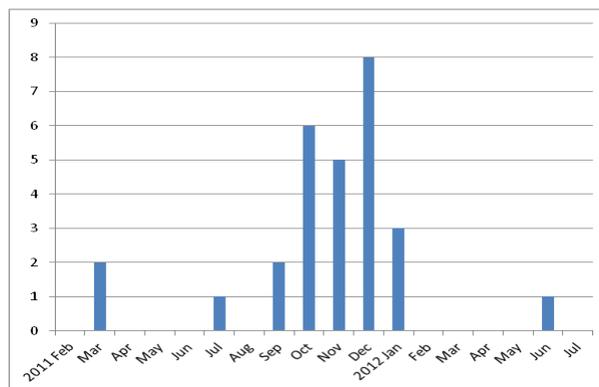
Specific etiology

Specific infectious agents were identified in a total of 64 (32.0%) out of 200 patients with respiratory tract infections by RT-PCR assay as follows: respiratory syncytial (RS) virus, 29; *M. pneumoniae*, 17; metapneumovirus, 7; adenovirus, 5;influenza A virus, 3; and influenza B virus, 3. Although RS and Influenza-positive samples were mainly obtained in winter time, several *M. pneumoniae*-positive samples were obtained in summer time (Figure 5a-f).

DISCUSSION

Acute respiratory infections, mostly caused by viruses and *M. pneumoniae*, are the most common illness experienced by healthy children worldwide as a major impact on health (25). Upper respiratory tract infections such as acute pharyngitis are prevalent in infants and children and continue to be common in young adults. Infants and children may have 3–8 episodes of common cold annually which lead to complications such as acute otitis media, asthma exacerbation, and lower respiratory tract infections. Although pneumonia, bronchitis, and bronchiolitis occur much less frequently, they cause higher morbidity associated with significant impact and greater healthcare costs. Approximately one third of infants develop lower respiratory tract infections in the first year of life. The incidence decreases to 5%–10% during school year, and 5% during ordinary young adult years.
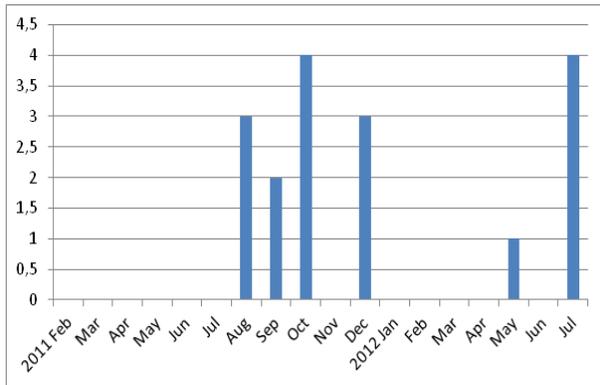
Figure 5c Cases of Mycoplasma pneumoniae (n=17).

Figure 5d Cases of Influenza B (n=3).

Figure 5a-f. Time distribution of Etiologic Agents by RT-PCR assay.

Figure 5a-f. Time distribution of Etiologic Agents by RT-PCR assay.

Influenza A and B, RS virus, parainfluenza virus, adenovirus, rhinovirus, human metapneumovirus, and coronaviruses are common viruses of respiratory tract (26,27,28). Clinical significance of recently discovered new viruses such as human bocavirus has yet to be elucidated (29,30). Clinical manifestations of viral infections of respiratory tract overlap among those caused by various other viruses. Effective antiviral treatment for respiratory virus infections is only available for influenza (26). Rapid diagnosis of respiratory viral infections is an integral part of health care. Accurate diagnosis of specific respiratory virus infection can affect patient management and help prevent secondary spread of the infection. Rapid viral diagnosis can result in discontinuation of unnecessary antibiotics and antiviral agents, reduction of costs related to reduction of unnecessary medical investigations, and shortened hospitalization (1).

A variety of microbiological assays have been applied to identify etiological agents from patients with respiratory tract infections. Although methods to determine etiological agents such as Gram's stain, isolation by tissue culture from clinical specimens and serum antibody detection have been widely utilized, due to the absence of a widely applicable international reference standard, none is indisputably predictive of the true cause of upper respiratory tract infections. Serological assays, antigen detection, RT-PCR study and isolation of the organism are used to determine viral etiology. Our results confirm many features of the etiology of lower respiratory tract infections that have been demonstrated in previous stud-
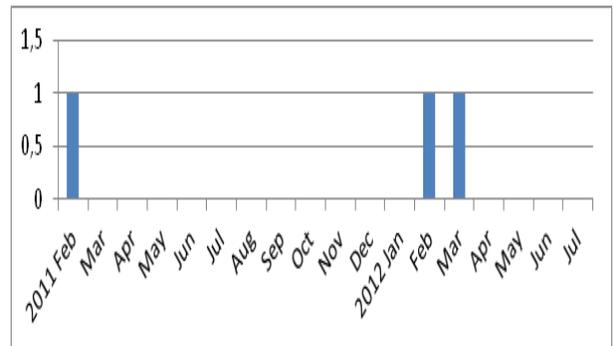
ies, in particular the high proportion of patients with mycoplasmal and viral infection (31,32). Although more information is needed about outpatients who are estimated to compromise more than half of the pediatric patients with community-acquired pneumonia or bronchitis, most etiologic studies have been conducted in hospitals in industrialized countries or in clinics for outpatients in developing countries.

The significance of mixed or poly-microbial etiology has¤increasingly been appreciated in the literature, particularly in studies that apply new, sensitive diagnostic methods to identify infection with *Mycoplasma, Chlamydia* and viruses (34). *S. pneumoniae* and *M. pneumoniae* were the major etiological causes of community-acquired pneumonia in children in previous studies, together accounting for up to 60% of cases (35). It is suggested that *M. pneumoniae* may predispose to secondary bacterial or viral infection.

The etiological profile among our hospitalized children accords well with the combined information from previous studies of community-acquired pneumonia and bronchitis in essentially unselected adult patients admitted to hospital. The divergent results are partly related to endemic and epidemic causes and partly to the microbiological methods. Unlike most studies of lower respiratory tract infections, the etiology remained unidentified in a relatively small proportion of the total cases in the present study. The hospitalization rate among these patients was not higher suggesting that most of the unidentified organisms caused more severe disease.
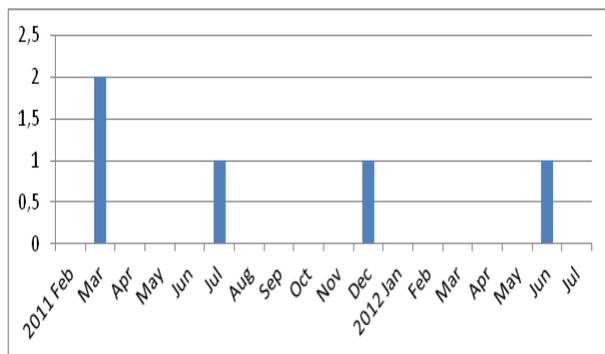
Figure 5e Cases of Adenovirus (n=5).

Figure 5a-f. Time distribution of Etiologic Agents by RT-PCR assay.

Figure 5f Cases of Metapneumovirus (n=7).

Figure 5a-f. Time distribution of Etiologic Agents by RT-PCR assay.

RS virus sometimes cause pneumonia in young infants, but more typically cause bronchitis or bronchiolitis. *M. pneumoniae* infections are common in school-aged children and are estimated to cause half of the cases or more (3,36). The etiological findings for younger adult patients resembled those for the older children enrolled in our present study; the proportions of *Mycoplasma* and RS virus infections were, however, higher especially among children less than 5 years of age.

To investigate the etiology of *M. pneumoniae* infections we conducted a prospective study covering the total pediatric population in Hokkaido, Japan (31,32). Paired sera for serologic assays were available for more than half of the cases (n = 921; 398 as pneumonia and 523 as bronchitis). The nasopharyngeal swabs were also collected for isolation and PCR study. *M. pneumoniae* was identified in 174 (43.7%) out of 398 patients with pneumonia and was identified in 78 (14.9%) out of 523 (86.2%) patients with bronchitis. Mycoplasmal infections were seen even in patients less than 5 years of age. Although two genetically distinct types of *M. pneumoniae* are known, variants of each also exist. Real-time PCR high-resolution melt genotyping assay was applied to identify clinical variants which may provide greater insight into the genetic distribution of *M. pneumoniae* strains (33). Generation of antigenic variation by DNA recombination may occur in clinical isolates (32).

For most respiratory viruses, the delay in transport of clinical specimens to the diagnostic laboratory is usu-
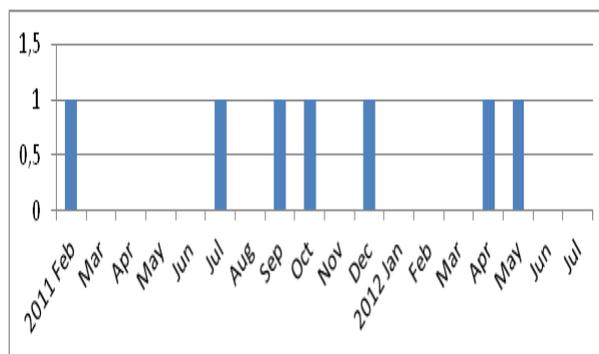
ally the major factor in both slowness of the process and a failure to make an appropriate clinical diagnosis. It should be possible to diagnose virus infections rapidly by electron microscopy and culture. It is necessary to make the diagnosis of respiratory tract virus infection more reliable and speedier. Molecular probes, and gene amplification, overcome these problems and have become routine standard technologies in the diagnostic setting (37).

Molecular biological assays have the advantages over conventional tissue culture methods of greater speed, specificity and sensitivity (1). They may be the only applicable techniques as many micro-organisms are fastidious and cannot be easily grown for phenotypic analysis in the ordinary laboratory. Apart from detection, molecular methods are now becoming standard for establishing quantitative estimates, particularly with regard to monitoring treatment, and resistance to therapy.

Detection of mRNA by RT-PCR has been successfully applied to the differential diagnosis of active viral infections. In RT-PCR, amplification (as detected by an increase in fluorescence) and analysis occur simultaneously. RT-PCR is well suited for quantitative analysis, but currently available commercially kits for detection of respiratory virus nucleic acids have only qualitative claims. RT-PCR instruments are limited in the number of emission channels available for multiplexing. Commercially available multiplex RT-PCR kits are generally limited to the detection of four or fewer nucleic acid targets (38). One of the targets is usually an internal control. FDA-cleared

RT-PCR assays are currently available for the detection of influenza A (including subtypes) and influenza B viruses, RSV, parainfluenza viruses 1, 2, and 3, and human metapneumovirus. Studies have demonstrated that molecular amplifications methods including real-time RT-PCR provide the most sensitive detection of respiratory viruses (17,18).

Diagnostic system used in the clinical evaluation of a rapid diagnostic reagent by immuno-chromatography has helped real rapid diagnosis in infections associated with such as influenza virus, RS virus, adenovirus, and *Streptococcus* from the superior speed and simplicity (8,9,10). Considering the time, difference in specificity and sensitivity comparisons with RT-PCR assay for detection of virus measles genome, immuno-chromatography was usually utilized from the beginning of the disease in general medical clinics (39).

Compared with RT-PCR assay, the sensitivity of the lateral flow-based immuno-chromatography rapid diagnostic reagent system was also much higher than that of other commercially available kits (11,12). The high sensitivity of the diagnostic test kit was confirmed by influenza A specimens that were confirmed by RT-PCR. As compared to influenza virus low titer of other viruses exist in the nasopharynx depending on time of specimen collection, the diagnostic reagent lower sensitivity than that of the RT-PCR assay. In this study high viral load time (2 to 4 clinical days after onset) in specimens has been achieved with regard to detection of measles virus. A further sensitivity is required including the purification of monoclonal antibody to the general practical level.

Considering the time, difference in specificity and sensitivity comparisons with RT-PCR assay, immuno-chromatography can be usually utilized from the beginning of the infectious diseases in general medical clinics. From the results of this study it can be applied as simple rapid diagnostic methods for pediatric respiratory infections in general clinical practice.

During the past several years, improvements are noticed in respiratory virus diagnostics, from novel specimen collection instruments to highly sensitive and multiplexed nucleic acid amplification tests. With the expanding list of antigen and molecular-based tests, it is now possible for laboratories to offer comprehensive testing for respiratory viruses without even performing virus isolation. Current diagnostic options will include antigen, molecular, and culture-based methods. It is important that diagnostic virologists and clinicians understand these characteristics and limitations.

In conclusion, the proportion of patients with *Mycoplasma* infections did not increase and that of patients with RS and influenza A viruses decreased with age, but for each age group, the etiological profile was completely different between inpatients and outpatients. *Mycoplasma* infections were seen even in patients less than 5 years, and RS and influenza A virus infections in patients more than 5 years of age. The results of our study confirm the importance of *M. pneumoniae* and RS virus in the etiology of community-acquired respiratory infections in Japanese children.

Further study overlapping on clinical specimen types, collection methods, collecting time and times of collection with diagnostic kits currently available for adenovirus, *Streptococcus* and influenza virus will improve diagnostic value (7). Also rise of serum IgM antibody is a biological reaction due to infections (40), the sensitivity and specificity of difference of the lateral flow-based immuno-chromatography and had been estimated in this study.

Conflict of interest

No commercial relationship or potential conflict of interest related to the submission of this manuscript.

Acknowledgments

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