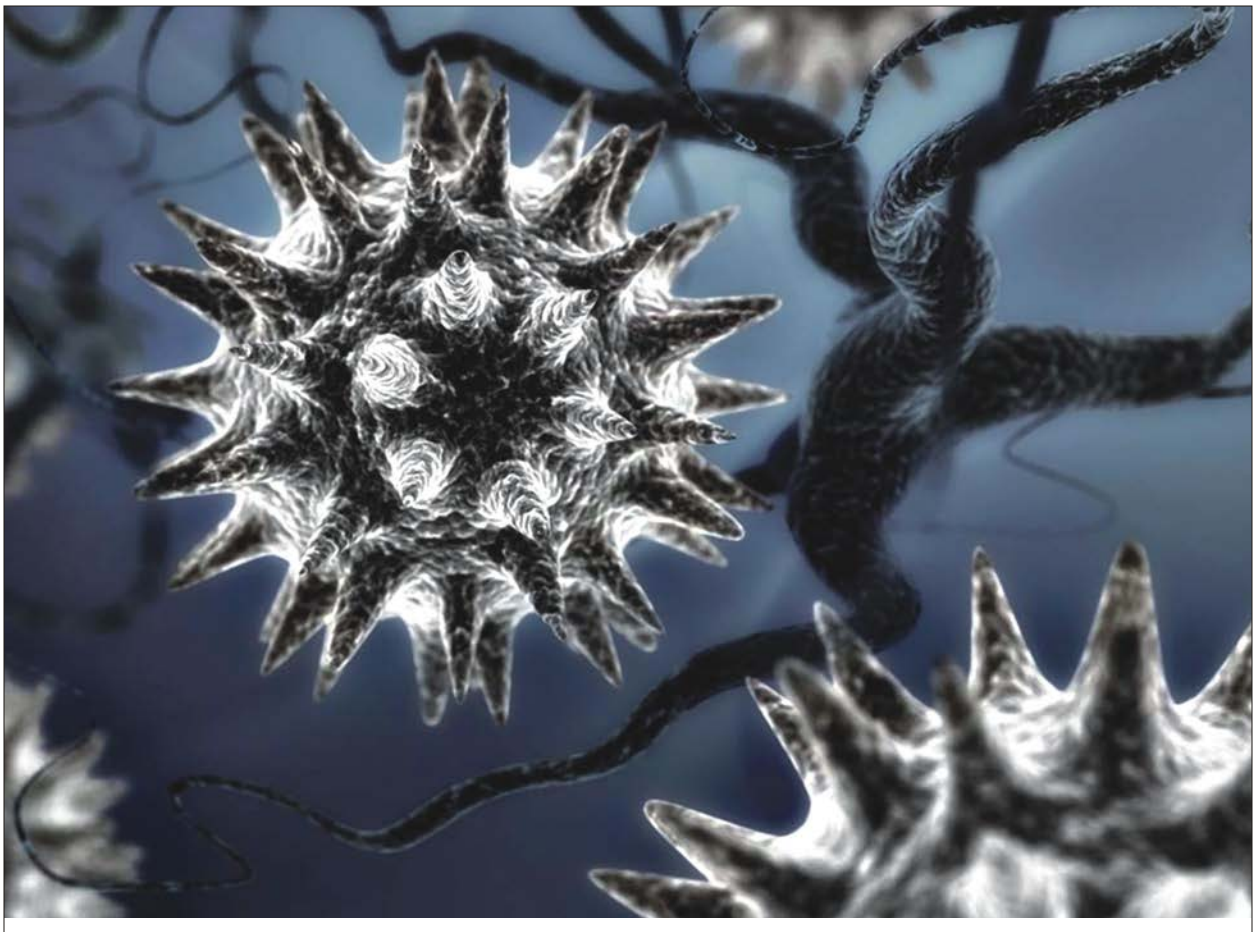

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Prevalence of Respiratory Syncytial Virus Infection in Riyadh During the Winter Season 2007-2008 and Different Risk Factors Impact

[1,3]Fahad N. Al-Majhdi, [2]A. Al-Jarallah, [1]M. Elaeed, [1]A. Latif,
[4]L. Gissmann and [1]Haitham M. Amer
[1]Department of Botany and Microbiology, College of Science,
[2]Department of Pediatric, College of Medicine, King Saud University,
[3]Center of Excellence in Biotechnology Research, College of Science,
King Saud University, Riyadh 11451, Kingdom of Saudi Arabia
[4]German Cancer Research Center, Program Infection and Cancer,
69120 Heidelberg, Germany

Abstract: In the current study, two RT-PCR systems for detection (mono-specific) and typing (duplex) of RSV in clinical samples have been developed and validated. The two assays were exploited for studying the dissemination of RSV infection in Riyadh, Saudi Arabia during the winter season 2007/2008. Furthermore, the effect of certain risk factors, including gender, age and disease form, on extend and impact of the infection was analyzed. Of two hundreds collected samples, 70 (35%) were positive for RSV infection, among which 40 (57.1%) were type A and 30 (42.9%) were type B viruses. This investigation is the first that describes the existence of both RSV subtypes in co-ordinance in Saudi Arabia children, with slight dominance of type A viruses. By analyzing the effect of risk factors on the disease epidemiology we confirmed that males, patients at young age and asthmatics are more susceptible for RSV infection and disease progression.

Key words: Respiratory syncytial virus, RT-PCR, typing, risk factors, Saudi Arabia

INTRODUCTION

Respiratory Syncytial Virus (RSV) is a single stranded, non-segmented RNA virus of negative-sense, which belongs to the Pneumovirinae subfamily of the Paramyxoviridae family, Order *Mononegavirales* (Riffault *et al*., 2006; Dimmock *et al*., 2007). It is recognized as the most frequent cause of severe lower respiratory tract infections in humans, particularly in young children, infants and immunocompromised individuals (Couch *et al*., 1997; Han *et al*., 1999; Falsey *et al*., 2005). According to the World Health Organization (WHO), RSV is responsible for approximately 64 million cases and 160,000 deaths every year worldwide. Respiratory syncytial virus infects more than 65% of infants during the first year of life and nearly all children experience one or more RSV infections by the end of the second year (Glezen *et al*., 1986). Hospitalization rates of infants are usually variable with the setting, with several developed countries having rates of 0.1-2% (Fisher *et al*., 1997). These rates

Corresponding Author: Dr. Fahad N. Almajhdi, Department of Botany and Microbiology, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Kingdom of Saudi Arabia
Tel: +966-4677778 Fax: +966-4675833

tend to be significantly higher for children in lower socioeconomic groups and in developing countries (Boyce *et al*., 2000; Hoffman *et al*., 2004). Mortality due to RSV infection are usually uncommon but highly significant in infants suffering from chronic lung diseases and congenital heart disease reaching up to 40% (Kim *et al*., 1973; Fixler, 1996) in immunocompromised individuals it can reach 80-100% (Whimbey *et al*., 1996).

Respiratory syncytial virus has a single serotype with two distinct antigenic subgroups A and B identified by panels of monoclonal antibodies (Anderson *et al*., 1985; Mufson *et al*., 1985). Sequence analysis of representatives of the two subgroups showed that they share high nucleotide and amino acid identity with a considerable divergence among structural proteins like G, M2-2, SH and F (Johnson *et al*., 1987; Collins *et al*., 1990). Some studies suggest that subgroup A viruses are more virulent than subgroup B viruses and replicate to higher titers in the respiratory tract (Walsh *et al*., 1997). Nevertheless, viruses from both subgroups are usually co-circulating during an epidemic with an alternating pattern regarding the predominant subgroup every 1-2 years (Peret *et al*., 1998; White *et al*., 2005). Therefore, characterization and typing of the circulating RSV strains became a necessary tool for the epidemiological investigations, a matter that enables better disease control strategies (Barr *et al*., 2000).

Although, the effect of RSV infection in children is well understood, the need for accurate and reliable virus detection and typing methods is still an urgent issue. Culture methods are traditionally recognized as the gold standard for virus isolation and diagnosis, however they are not generally acceptable in every-day practice due to the required time, effort and costs (Welliver, 1988). Serological techniques such as direct immuno-fluorescence and antigen capture ELISA may provide quicker results but they lack the desired sensitivity and sometimes specificity (Johnson and Siegel, 1990; Kuypers *et al*., 2006). During the last two decades, molecular biology based-assays, particularly end-point and real-time PCR proved their ability to overcome these limitations and provided simple and high-throughput amenable tools in detection, typing and quantification of many respiratory viral agents including RSV (Osiowy, 1998; Falsey *et al*., 2002; Bellau-Pujol *et al*., 2005).

In the current study, two different RT-PCR systems were developed and optimized for detection (mono-specific) and typing (Duplex) of RSV in clinical samples. The developed assays were utilized in screening of 200 nasopharyngeal aspirates (NPAs) collected from hospitalized children with acute respiratory tract infections in Riyadh, KSA. Different risk factors were analyzed for their influence in disease predisposition.

MATERIALS AND METHODS

Specimens

Nasopharyngeal aspirates were collected from 200 children aged from one month to three years and hospitalized with suspected acute respiratory tract infections during the period extended from late autumn of 2007 till spring of 2008. The aspirates were taken by trained nurses at King Khalid University Hospital, Riyadh, Saudi Arabia, who inserted a sterile catheter into the nasal cavity of patients to a depth of 5-7 cm and drawn back while applying gentle suction using an electronic suction device (Heikkinen *et al*., 2002). Specimens were transported in Minimal Essential Medium (MEM) supplemented with 500 U penicillin and 500 µg streptomycin per mL to the Research Central Laboratory, College of Science, King Saud University, Riyadh, where they processed, tested and stored at -80°C.

RNA Extraction

Viral RNA was isolated from fresh and frozen samples using QIAamp Viral RNA Mini Kit (Qiagen GmbH, Hilden, Germany). Each sample aliquot was used only once to avoid the

loss of viral genomic material during repetitive freezing and thawing. The viral RNA extraction kit combines the selective binding properties of a silica gel membrane with the speed of microspin technology. The procedure was conducted utilizing 140 µL sample volumes according to the manufacturers instructions. Purified RNA was eluted from QIAamp columns in 60 µL elution buffer and stored at -20°C for use in mono-specific and duplex RT-PCR assays.

Primer Selection and Synthesis

The primers used for identification and typing of RSV strains were selected and optimized on the basis of GeneBank data of a wide variety of human RSV isolates. A primer pair that amplifies a 279 bp fragment of Nucleocapsid (N) gene of RSV was completely conserved for both RSV subtypes and used in mono-specific RT-PCR (Cane and Pringle, 1991). Two different primer sets were utilized for subtyping of RSV strains in a duplex RT-PCR: one for type A RSV that amplifies a 413 bp fragment of Fusion (F) gene and the other amplifies 149 bp fragment of the type B RSV N gene (Templeton *et al*., 2004) (Table 1).

First Strand Synthesis

The viral RNA extract was reverse transcribed using Sensiscript® Reverse transcription kit (Qiagen GmbH, Hilden, Germany) according to the manufacturers guidelines. A reaction mixture contained 5 µL of template RNA, 2 µL of 10x buffer RT, 2 µL of 5 mM dNTPs mix, 200 µM of random primers, 10 U of RNase inhibitor and 1 µL of sensiscript® Reverse Transcriptase was prepared and completed to 20 µL total reaction volume by RNase free water. The mixture was incubated for 60 min at 37°C and then cooled to 4°C till use in mono-specific or duplex PCR. The synthesized cDNA products were analyzed by agarose gel electrophoresis for further confirmation.

Mono-Specific and Duplex PCR

Two RSV-specific PCR systems were developed and optimized in the current study: the first utilized universal N gene primers and aimed for detection of RSV infection in suspected samples (Mono-specific RSV-PCR), while the second utilized the two primer sets that identify subtypes A and B of RSV simultaneously in a single reaction tube (Duplex RSV-PCR). Both PCR reactions were set up using Multiplex PCR kit (Qiagen, Hilden, Germany) with specific technical adaptations for ideal amplification results. A final reaction volume of 50 µL containing 25 µL of 2×Qiagen Multiplex PCR Master Mix, 2 µM of each specific primer (universal N gene primers for mono-specific PCR and RSV-A and RSV-B primer sets for duplex PCR), 2 µL of the cDNA product and 21 µL of RNase free water was prepared for each

Table 1: Oligonucleotide primers for mono-specific and duplex RT-PCR

Primer name	Target gene	Primer sequence	PCR product (bp)	Reference
RSV-Forward	N gene (Universal)	5`- GGA ACA AGT TGT TGA GGT TTA TGA ATA TGC-3`	279 bp	Cane and Pringle (1991)
RSV-Reverse		5`- TTC TGC TGT CAA GTC TAG TAC ACT GTA GT-3`		
RSV-A-Forward	F gene (Subtype A)	5`- GTG TAA CAA CAC CTT TA AGCA CTT ACA TG -3`	413 bp	Templeton *et al*. (2004)
RSV-A-Reverse		5`- GTA ATG TTA AAC TGT TCA TAG TGT CAC -3		
RSV-B-Forward	N gene (Subtype B)	5`-AAT GGA AAA GAA ATG AAA TTT-3`	149 bp	
RSV-B-Reverse		5`-GGA GAA TCA TGC CTG TAT TC3-3`		

sample. Sample tubes were incubated in a thermal cycler (Primus 96plus, MWG AG Biotech, Ebersberg, Germany) for one cycle at 95°C for 15 min followed by 35 cycles of 94°C for 30 sec, 50°C for 90 sec and 72°C for 90 sec and finally one cycle of 72°C for 10 min. The PCR products were separated in 1.5% agarose gel containing 0.5 μg mL^{-1} ethidium bromide and corresponding bands were identified as compared with 1 kbp DNA ladder (Invitrogen, San Diego, CA, USA) using an image analysis system (IMAGO Compact Imaging System, B and L, USA).

RESULTS AND DISCUSSION

Seasonal epidemics of RSV infection cost several thousands of lives throughout the world every year, primarily among children with underlying cardiac, pulmonary and immunologic disorders (De Vincenzo, 2007). A wide range of risk factors either predispose or complicate the infection status including age, gender, premature birth, passive smoking, lack of pure breast feeding, disease form and medical history of respiratory diseases and allergy (Welliver, 1988; Meqdam and Subiah, 2006).

In Saudi Arabia, only few reports described the prevalence of RSV infection in sporadic districts of the kingdom including Riyadh, Al-Quassim and Abha (Jamjoom *et al*., 1993; Bakir *et al*., 1998; Al-Hajjar *et al*., 1998; Al-Shehri *et al*., 2006; Meqdam and Subiah, 2006). These reports covered short periods of time extending from 1991-1996 and 2003-2004. From these available data it appears that RSV is the main causative organism involved in acute respiratory tract infections and hospitalization of young children in Saudi Arabia with an incidence range of 28.5-54%. However, the use of serological and virological assays of limited specificity and sensitivity may impact the validity of these results. Currently, we are lacking important information regarding the situation of RSV infection, the predominant subtypes and impact of different risk factors on the disease status. No virus isolate is available for characterization and vaccine preparation.

Since, RSV infection is highly contagious and usually requires hospitalization, rapid and accurate diagnosis of the virus infection is very important for preventing nosocomial infections, a matter that profoundly decreases extent and severity of the infection in a community (Mlinaric-Galinovic *et al*., 1996; Deiman *et al*., 2007). Moreover, it enables the application of appropriate patient management, control measures and helps to avoid the unnecessary use of antibiotics (Wilson *et al*., 2002; Slinger *et al*., 2004; Kafetzis, 2004).

With the advance of molecular biology and development of more specific and sensitive techniques to test for viral pathogens, especially PCR, it is now possible to examine the prevalence of different viruses implicating in respiratory tract infection more accurately (Kitchin, 1990; Nadder and Langly, 2001; Espey *et al*., 2006). Therefore, in the current study we optimized a standard two-step RT-PCR assay for direct diagnosis of RSV in clinical samples. Despite the preference of single-step RT-PCR in routine diagnostic approaches (handling errors, contamination risks and work load issues are significantly decreased) the two-step RT-PCR offers better flexibility and quality control potential. In addition, lower primer-dimer formation and availability of more stable cDNA libraries for sample archiving and epidemiological studies are major advantages of the two-step approach (Vandesompele *et al*., 2002).

The developed RT-PCR assay makes use of a primer set that amplifies a fragment of 279 bp in the nucleocapsid gene sequence (Fig. 1). The potential of the N gene for development of such an assay relies on the sequence conservation and high level of expression accounting for better sensitivity and specificity (Cane and Pringle, 1991; O`Shea

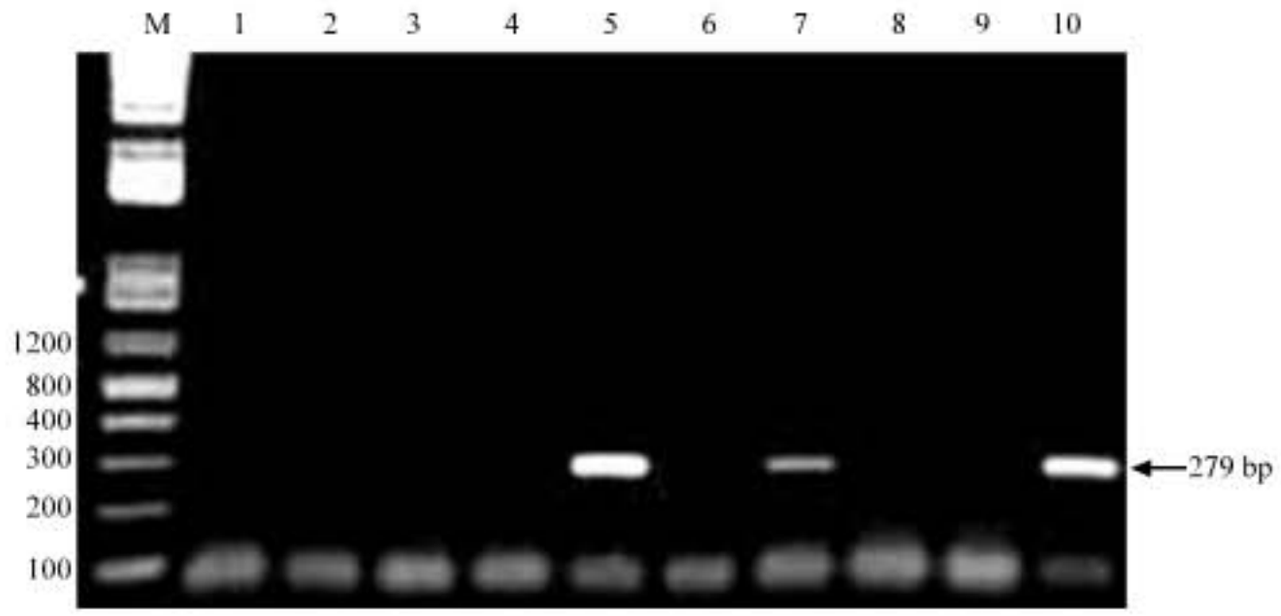

Fig. 1: Mono-specific RT-PCR products of selected NPA samples for detection of HRSV as shown by agarose gel electrophoresis: Lane M represents 1 kbp DNA molecular weight ladder plus, Invitrogen; Lanes 5, 7 and 10 show positive amplification of a 279 bp fragment of HRSV-N gene sequence and Lanes 1, 2, 3, 4, 6, 8 and 9 show negative results

and Canea, 2004; Yamada *et al*., 2004; Dewhurst-Maridor *et al*., 2004). To further investigate the specificity of the assay, different human respiratory viral agents were tested in parallel. No cross-reactivity could be observed for Influenza A and B viruses, Parinfluenza-2 and 3, Human Metapneumoviruses and Measles virus, indicating that the assay was specific for RSV.

Respiratory syncytial virus subtypes A and B occur either simultaneously or alternate during the annual epidemics of RSV infection (Papadopoulos *et al*., 2004). The available information concerning the clinical severity, the geographical distribution and the chronology of either subtype is still controversial (Walsh *et al*., 1997; White *et al*., 2005). Typing of RSV-positive samples on temporal and spatial bases will shed more light on the epidemiology of RSV infection and consequently enable the design of comprehensive and long-term control strategies. Therefore, we developed a duplex RT-PCR assay for the detection of both RSV subtypes, using primer pairs that specifically differentiate between type A and B viruses (Fig. 2). The sensitivity, specificity and reproducibility of the assay was evaluated and confirmed by the standard means.

For studying the prevalence of RSV in Riyadh, Saudi Arabia, during the winter season of 2007-2008 and implication of some risk factors (e.g., gender, age and disease form) on the infection status, we further exploited the aptitude of the two developed RT-PCR systems for detection and typing of RSV in clinical samples. Since, conduction of a investigation that covers all of Riyadh city proved to be too difficult, we focused on the collection of samples from children registered at King Khalid University Hospital, which is an educational hospital that accepts patients from the whole Riyadh city and many nearby districts. Of 200 tested NPA samples, 70 were positive for RSV infection (35%) (Table 2). This ratio is well in line with other studies that identify RSV as the main causative agent in at least one quarter of respiratory infection cases of hospitalized children in Saudi Arabia and worldwide (Holberg *et al*., 1991; Bakir *et al*., 1998; Al-Hajjar *et al*., 1998; Al-Shehri *et al*., 2006; Hu *et al*., 2003; Mentel *et al*., 2005).

Typing of the positive samples using duplex RT-PCR indicated that 57.1% were type A viruses and 42.9% were type B. These results validate the implication of both virus subtypes

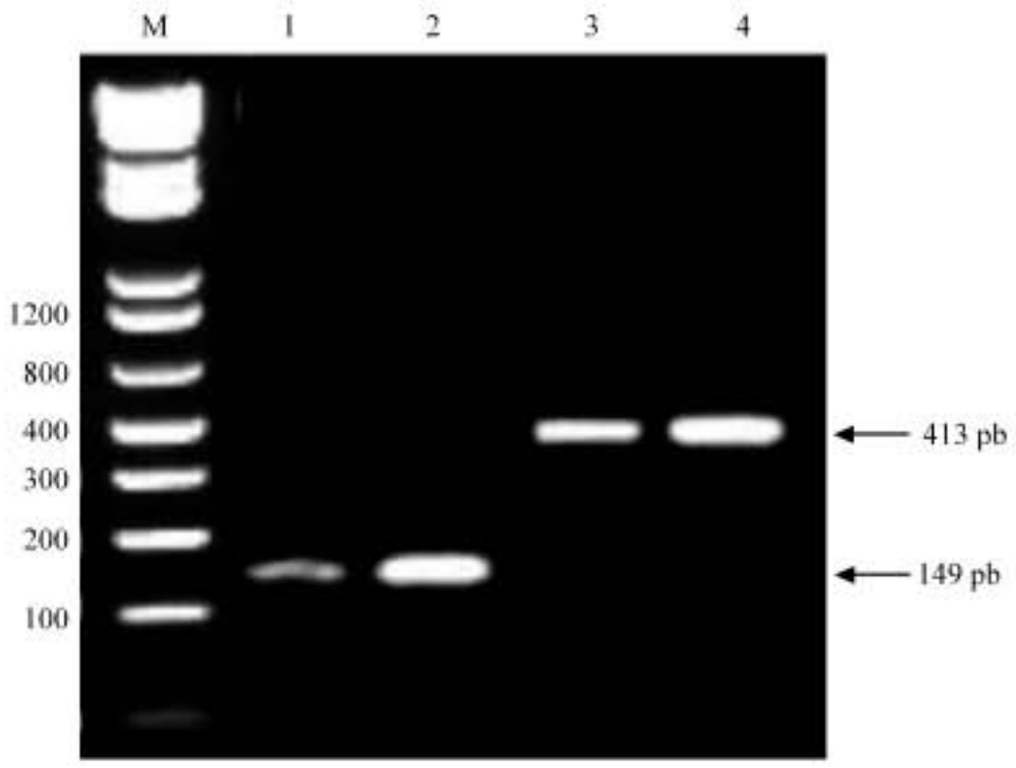

Fig. 2: Duplex RT-PCR products of four selected RSV-positive NPA samples using two primer sets that identify types A and B viruses simultaneously, shown by agarose gel electrophoresis: Lane M represents 1 Kbp DNA molecular weight marker; Lanes 1 and 2: type B RSV viruses lanes 3 and 4: type A RSV viruses

Table 2: Influence of different risk factors (Gender, age and disease form) on the prevalence of RSV and its major subtypes in hospitalized children under study

Risk factors	RSV subtype A No.* (%)	RSV subtype B No. (%)	RSV positive No. (%)	Total No.
Gender				
Male	21 (20.7)	17 (16.6)	38 (37.3)	102
Female	19 (19.4)	13 (13.3)	32 (32.7)	98
Age group				
0-6 months	10 (18.2)	8 (14.5)	18 (32.7)	55
6-12 months	20 (43.5)	11 (23.9)	31 (67.4)	46
12-24 months	8 (16.6)	5 (10.4)	13 (27)	48
24-36 months	2 (3.9)	6 (11.8)	8 (15.7)	51
Disease form				
Upper RTI**	5 (29.4)	3 (17.6)	8 (47)	17
Lower RTI	28 (16.7)	23 (13.7)	51 (30.4)	168
Asthma	7 (46.6)	4 (26.7)	11 (73.3)	15
Total	40 (20)	30 (15)	70 (35)	200

*No.: Number, **RTI: Respiratory tract infection

in RSV infection of Saudi Arabia children during the winter season 2007-2008 with a slight dominance of type A viruses. No distinct association between the virus subtype and clinical outcome could be observed from our data, since, both subtypes were identified in cases of different disease categories with comparable ratio.

The study was also designed to evaluate the effect of different risk factors like gender, age and disease form on the extent and impact of RSV infection in Saudi Arabia children. For accomplishing such purpose, the samples used in this study were collected in a balanced manner concerning each of these risk factors. The only exception was the disease form category which includes higher frequency of patients with lower respiratory tract infection since most of the hospitalized children are basically suffering form bronchiolitis and/or pneumonia.

Regarding the gender risk factor, the results obtained delineate that male patients (No. = 38/102; 38.7%) are at a slightly higher risk than female patients (No. = 32/98; 31.3%), which appears in agreement with many previous investigations (Wang and Law, 1998;

Iwane *et al*., 2004; Gerna *et al*., 2008). This phenomenon was explained by Meissner (2003) in which they indicated that the airways of young males are shorter and narrower than those of young females leading to a worse evolution of the disease. Male being of higher risk applies for both virus subtypes A and B (Table 2).

Respiratory Syncytial Virus infection is usually associated with children younger than 5 years and particularly younger than one year (Wang and Law, 1998). The age-distribution of RSV illness appears similar throughout the world; however, there is no documented data demonstrating such similarity in Saudi Arabia children. In this study, we grouped the samples into four main age groups: 0-6 months; 6-12 months; 1-2 years and 2-3 years. The results showed that 70% of the RSV-positive samples were collected from children younger than one year and then the incidence decreases by developing in age (18.6% during the second year and 11.4% during the third year). The same situation exists for both subtypes A and B with significant higher preference of type B to less younger ages.

Although, RSV is associated with all aspects of respiratory tract illness in children, it is highly incriminated in most Lower Respiratory Tract (LRT) affections as well as wheezing and asthma (Kim *et al*., 1973). In the present study, the relative implication of RSV in LRT illness (28.5%) was expected and nearly identical to the international rates (Holberg *et al*., 1991). However, the remarkable observation is the significant higher ratio of RSV-positive samples in asthmatic patients (73.3%). This supports the idea that RSV is the predominant pathogen involved in wheezing and asthma among children in preschool age (López-Pérez *et al*., 2009). On the other hand, we cannot conclude that the increased positive reactivity of the samples collected from patients with Upper Respiratory Tract (URT) affections (47%) has any significant value since UTR affected patients rarely underwent hospitalization and number of test samples were really restrictive (Table 2).

In conclusion, the present study exploited two RT-PCR systems for detection and typing of RSV in 200 clinical samples collected from Riyadh, Saudi Arabia during the Winter season 2007-2008. Both RSV types were detected in the test samples with slight dominance of type A. Risk factors including male gender, young age (less than one year) and asthma may have the potential that favors RSV infection in Saudi Arabia children. Further utilization of the developed assays in tracking the RSV infected cases on spatial and temporal bases will provide significant information about the virus infection, disease progression and spread.

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