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Direct Immunofluorescence Detection of Respiratory Syncytial Virus among Egyptian Infants under Two Years of Age with Acute Lower Respiratory Tract Infection

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MGB, EAE and MMA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors RAEHAED and NGB managed the analyses of the study. Author MGES managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim of the Work: evaluation of direct fluorescent assay (DFA) in the detection of RSV in nasopharyngeal secretions of infants with acute LRT infections and revealing the rate of RSV infection in these patients.
Patients and Methods: This study included 100 infants less than two years of age who diagnosed as cases of acute LRT infections During the 6 month study period, from October 2013 till April 2014. Cases were presented as an outpatient clinic and/or were hospitalized in the pediatric hospital of Tanta University.

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Results: 20% of infants included in the study were positive for RSV by DFA. The results revealed a significant relationship between RSV infections and younger ages with mean age 3.5 months. The incidence of RSV infection was also significantly increased with bronchiolitis, wheezy chest, congenital heart disease, prematurity, family history of asthma and winter season. Conversely, there was an insignificant relationship between RSV infection and type of feeding and antibiotics consumption.
Conclusion: So, DFA can be used as an effective tool in screening large numbers of cases for rapid diagnosis of RSV infection. This can help in early treatment with specific anti-viral and avoid unnecessary use of antibiotics, where most of cases are treated as bacterial infections.

Keywords: Respiratory syncytial virus; nasopharyngeal aspirate; infant-direct immunofluorescencent assay.

1. INTRODUCTION

Respiratory infection is the primary cause of hospitalisation and morbidity in infants [1], of which a significant proportion are caused by viruses [2,3]. Human respiratory syncytial virus (RSV) is one of the major causes of acute lower respiratory tract infections (ALRTI) in infants below one year as well as in elderly and immunocompromised adults. The disease control centers consider RSV to be the "most common cause of bronchiolitis and pneumonia in infants under 1 year of age [4]. A recent meta-analysis suggests that up to 200 000 children aged younger than 5 years die worldwide each year because of RSV-related infections [5].

RSV is an enveloped, single-stranded RNA virus from the Paramyx-Oviridae family [6]. There are two major antigenic groups of RSV, A and B, based on monoclonal antibodies to the major structural glycoproteins G and F (7). Children initially infected with a group A RSV have relatively protected against group A infection, and reinfections are more likely to occur throughout life due to the heterologous group B RSV, which reflecting incomplete immunity to the virus [8].

The incidence of RSV varies significantly based on seasonality, with the majority of infections occurring in the winter months and lasting an average of 6 months especially in tropical and subtropical areas. However, its infection may occur around the year in equatorial areas [9,10].

RSV spreads easily by direct contact as it can remain viable for up to 5 hours on hands [11].The incubation period of RSV disease is estimated to be 5 days [12]. At the beginning of the illness, the virus replicates in the nasopharynx, and then spreads from the upper respiratory tract to the lower airways along the respiratory epithelium inducing cell fusion and syncytium formation [13].

The symptoms of RSV infections are variable; it may be mild symptoms that indistinguishable from common colds and minor illnesses with poor appetite and fever [14]. However, severe respiratory illness is more likely to occur in the premature or the immunocompromised infants that requiring hospitalisation. So, surveillance of respiratory viruses is important to predict seasonal epidemics, define patient risk groups as well as to describe the burden and characteristics of emerging viruses [15]. Currently, there are different laboratory methods can be used for specific diagnosis of RSV infection in respiratory secretion. Such as; virus isolation in tissue culture, detection of viral antigens by direct or indirect immunofluorescent assay (DFA/IFA) or by enzyme-linked immunosorbent assays (EISA) and the detection of viral nucleic acids by amplification assays, predominantly reverse transcription polymerase chain reaction (RT-PCR) [16]. However, DFA is considered the more rapid and the less expensive methods for routine surveillance of respiratory viruses [17].

Therefore, this study aimed to rapid detection of respiratory syncytial virus and identifies the rate of its infection in Egyptian infants below two years with clinical diagnosis of acute lower respiratory tract infections.

2. PATIENTS AND METHODS

2.1 Patients

This study was carried out on 100 infants less than two years of age presenting to the outpatient clinic and/or were hospitalized in the pediatric hospital of Tanta university. Those

infants were clinically diagnosed as acute lower respiratory tract infection with or without radiological confirmation.

Infants were excluded if they were over two years of age, or they were diagnosed as case of chronic lower respiratory tract infection, upper respiratory tract infection or confirmed laboratory diagnosis as non-viral cause of infection.

Written informed consent was obtained from a parent of each infant. This study was approved by the Ethics Committee of faculty of medicine, Tanta University.

During the 6 month study period, from October 2013 till April 2014, the enrolled infants were subjected to;

- **Complete history** taking from the parents including age, sex, onset, course and duration of illness, antibiotic therapy, type of feeding breast feeding or artificial, disease history as lower respiratory tract infection, congenital heart disease, wheezy chest, prematurity and family history of asthma.
- **Physical examination** included general examination, chest examination and plain x-ray to detect the presence of sign of respiratory distress (tachypnea, chest indrawing, grunting or cyanosis) and other chest findings as wheezing and crepitations.

2.2 Sample Collection and Preparation

Nasopharyngeal aspirates (NPA) were collected from participants within 5 days of illness using mucous extractor connected to suction apparatus with the patient head was extended back as far as possible while drops of sterile saline were applied into one nostril with a syringe. The catheter was placed through nostril to posterior nasopharynx, then gentle suction was applied and the catheter was slowly withdrawn. This procedure was repeated through other nostril for optimal sampling (1-2 ml). The specimens were delivered to the lab within 1 hour for processing (88). The specimens were centrifuged for 10 min at 2000 rpm. 3-4 ml of phosphate buffer solution (PBS) was added to each sediment with gentle mixing. One drop of the final suspension was placed on coated slide which were allowed for air drying. The slides were fixed with 10% formaline for 15 minutes at room temperature, following by twice rinsing with PBS. The slides were leaving for air drying completely before storing at -20°C until time of staining.

2.3 Direct Immunoflurescent Assay

The samples were returned to room temperature and incubated with 0.3% PBS/Triton X-100 for 10 min at 37°C, following by rinsing with PBS for 5 min. The unspecific binding of antibody was blocked by incubating with 3% Bovine Serum Albumin (BSA) in PBS for 1 h at 37°C. The slides were incubated with primary antibody (goat anti-RSV FITC, 1:100) for 1 h at 37°C. The slides were rinsed with PBS (2x5 min) and sterile distilled water. The slides were covered using vectashield mounting medium to be examined and photographed under fluorescence microscope. The specificity of the antibody reaction was verified by the absence of immunostaining in the negative control slide that treated with blocking serum without the primary antibody.

2.4 Statistical Analysis

The data were statistically analyzed using SPSS software (Statistical Package for the Social Sciences, version 16, SPSS Inc. Chicago, IL, USA). For quantitative data (as age of RSV positive infants), the range, mean and standard deviation were calculated. For qualitative data (as type of infant feeding), comparison between two groups and more was done using Chi-square test ($\chi 2$) and Fisher Exact test. For comparison between means of two groups, parametric analysis (Student t-test) was used (154). Data were presented as mean ± SD (standard deviation) with $p<0.05$ was considered statistical significant.

3. RESULTS

During the 6 month study period, nasopharyngeal aspirates were collected from 100 infants with Acute Lower Respiratory Tract Infections (ALRTI). All samples were processed for detection of Respiratory Syncytial Virus (RSV) by direct immunofluorescence assay (DIF). The study showed that 20 samples (20%) were positive for RSV from the total 100 samples. The immunostaining reaction of RSV positive infants revealed positive cytoplasmic reaction that was limited to the cytoplasm of single epithelial cells or it expressed in the infected cells and nearby cells with syncytium formation (Fig.1). The specificity of the antibody reaction was verified by the absence of immune staining in the

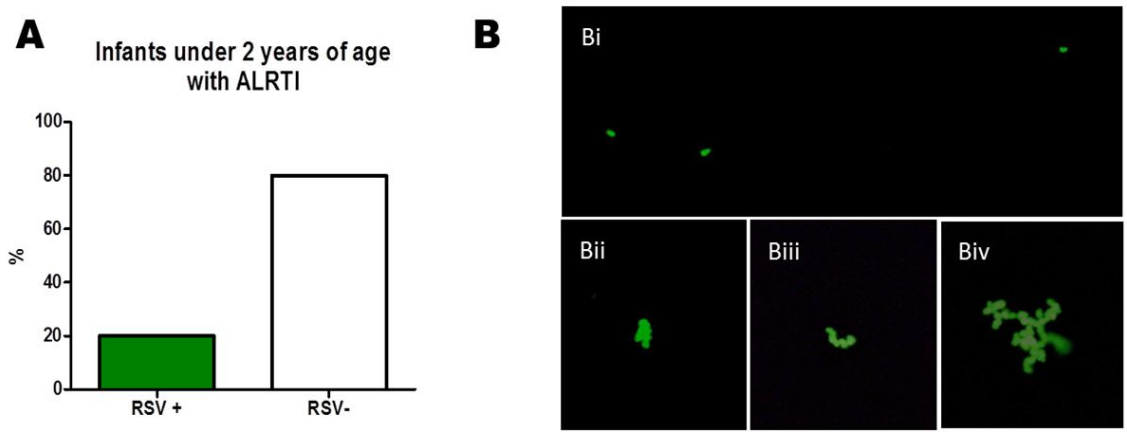

Fig. 1. Respiratory syncytial virus (RSV) infection. A: Percentage of infants under 2 years of age of acute lower respiratory tract infection (ALRTI) with and without Respiratory syncytial virus (RSV) infection. B:Immunofluorescence staining of nasopharyngeal aspirate with anti-RSV antibody showing positive cytoplasmic reaction (green color) either in separate epithelial cells (i) or in group of epithelial cells with different degree of syncytium formation (ii,iii and iv). (40X)

negative control slide that treated with blocking serum without the primary antibody.

The study showed that the age of RSV positive cases was below or equal to 6 months in 16 cases (80.0 %) and above 6 months in 4 cases (20.0%), while in RSV negative infants 8 cases (10.0%) were below or equal to 6 months in age and 72 cases (90.0%) were above 6 months in age with statistically significant (*P*=0.0001) between RSV infection and the young age (Table 1 & Fig. 2A). The mean age of RSV positive cases was 3.50 months while the mean age of RSV negative cases was 6.90 months with a statistically significant difference (*P*=0.002).

The study revealed that sex distribution of RSV positive infants was as the following 12 cases (60.0%) were male and 8 cases (40.0%) were females, however among RSV negative infants 56 cases (70.0%) were males and 24 cases (30.0%) were females with insignificant relationship (*P*=0.556) between sex and RSV infection as (Table 1 & Fig. 2B).

As regard the type of feeding of RSV positive cases, 5 cases (25.0%) received breastfeeding and the remaining 15 cases (75.0%) received artificial feeding but among RSV negative infants 39 cases (48.8%) received breastfeeding and 41 cases (51.2%) received artificial feeding with statistically insignificant relationship (*P* =0.096) between type of feeding and RSV infection (Table 1 & Fig. 2C).

The study revealed the relationship between family history of asthma and RSV infection. Between RSV positive cases 12 cases (60.0%) had a positive family history of asthma and 8 cases (40.0%) had a negative family history of asthma. Among RSV negative infants 25 cases (31.2%) had a positive family history of asthma and 55 cases (68.8%) had a negative family history of asthma with statistically significant relation (*P*=0.034) between family history of asthma and RSV infection (Table 1 & Fig. 2D).

About the clinical diagnosis, among RSV positive cases 16 cases (80.0%) were diagnosed clinically as bronchiolitis and 4 cases were diagnosed as pneumonia (20.0%), while among RSV negative infants 35 cases (43.8%) were diagnosed as bronchiolitis and 45 cases (56.2%) were diagnosed as pneumonia with statistically significant relationship (*P*=0.008) between bronchiolitis and RSV infection (Table 1& Fig. 2E).

Among RSV positive infants 15 cases (75.0%) received antibiotic therapy and 5 cases (25.0%) didn't receive any antibiotic therapy, however among RSV negative infants 58 cases (72.5%) received antibiotic therapy and 22 cases (27.5%) didn't receive any antibiotic therapy with insignificant difference (*P* =0.823) between the 2 groups as regard using antibiotics (Table 1 & Fig. 2F).

The study demonstrated the risk of any associated medical conditions on RSV infection. 4 cases (20%) out of RSV positive cases and 2 cases out of negative cases (2.5%) had CHD with statistically significant relationship (*P* =0.014) between CHD and RSV infection. 5 cases (25%) out of RSV positive cases and 4 cases (5%) out of negative cases were premature with statistically significant relationship (*P*=0.015) between prematurity and RSV infection. Also 10 cases (50%) out of RSV positive cases and 17 (21.2%) out of negative cases had recurrent wheezy chest with statistically significant relationship (*P*=0.021) between wheezy chest and RSV infection (Table 1 & Fig. 2G).

As regard seasonal distribution of RSV positive and negative cases, it was found that: in autumn season, 11 samples (21.6%) were positive out of 51 cases and 40 cases (78.4%) were negative. In winter season, 9 samples (31.0%) were positive out of 29 cases and 20 cases (69.0%) were negative. In spring season, no positive sample (0.0%) was found and 20 cases were negative (100.0%). There was statistically significant relationship (*P*=0.026) between winter season and RSV infection (Table 1 & Fig. 2H).

4. DISCUSSION

Human respiratory syncytial virus (RSV) is one of the most important respiratory pathogens and a major cause of acute lower respiratory tract infections (ALRTI) in infants below one year that is often requiring hospitalization. RSV infection is considered as a major health and economic problem due to high mortality rate of children under age 5 with expenditure of lot of money for health care of infected patients [18,19]. So the present study was carried out for rapid detection of RSV in nasopharyngeal secretions by direct immunofluorescence assay (DFA) in infants with ALRTI aiming to make rapid diagnosis of cases with detection the rate of RSV infection in these patients.

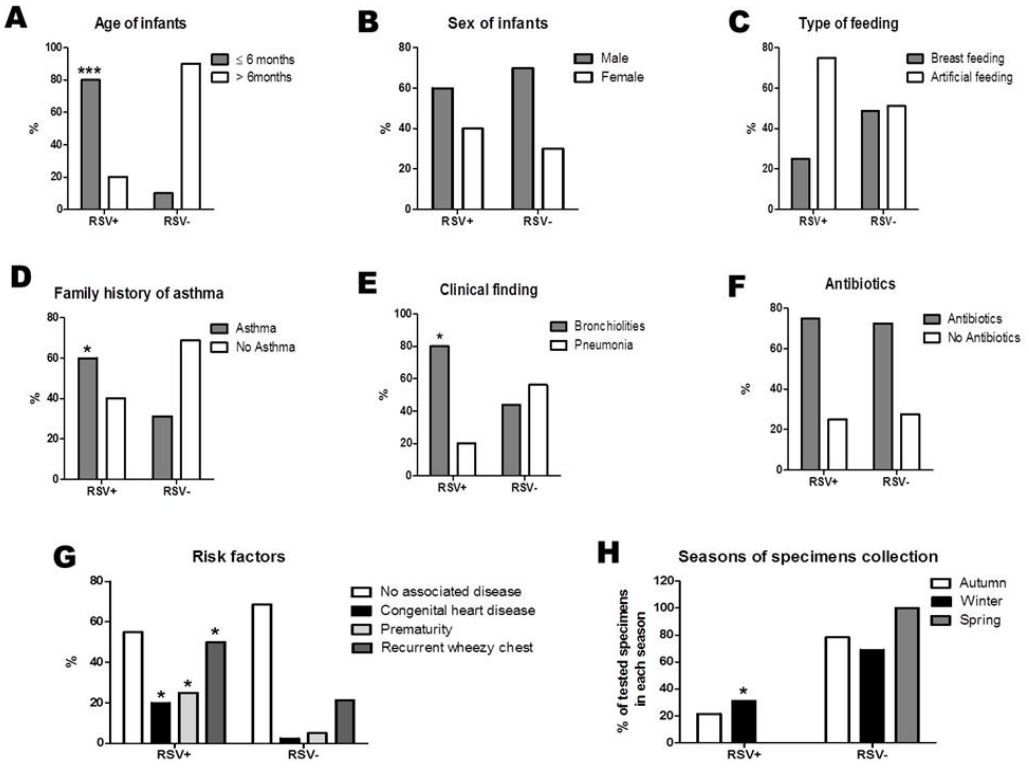

Fig. 2. Characteristic of Respiratory Syncytial Virus (RSV) positive and negative infants under 2 years of age with Acute lower respiratory tract infection regarding; (A) age, (B)sex, (C)type of feeding, (D)family history of asthma, (D)clinical finding, (F)receiving antibiotics, (G)risk factors and (H)main season of infection

**P<0.05, ***P≤0.0001*

Table 1. Percentage of infants under 2 years of age of acute lower respiratory tract infection (ALRTI) with and without Respiratory Syncytial virus (RSV) infection. * P< 0.05 is considering significant

Infant with ALRTI (n=100)			
Variable	**RSV-positive (n=20)**	**RSV- negative (n=80)**	***P* Value**
	n. (%)		
▪ **Ages in months**			0.0001*
≤ 6 months	16(80.0)	8(10.0)	
> 6 months	4(20.0)	72(90.0)	
▪ **Sex**			0.556
Male	12(60.0)	56(70.0)	
Female	8(40.0)	24(30.0)	
▪ **Type of feeding**			0.096
Breast feeding	5(25.0)	39(48.8)	
Artificial feeding	15(75.0)	41(51.2)	
▪ **Clinical finding**			
Bronchiolitis	16(80.0)	35(43.8)	0.008*
Pneumonia	4(20.0)	45(56.2)	> 0.05
Received antibiotic	15(75.0)	58(72.5)	0.823
▪ **Risk factors**			
Family history of asthma	12(60.0)	25(31.2)	0.034*
No associated disease	11(55.0	55(68.7)	0.370
Congenital heart disease	4(20.0)	2(2.5)	0.014*
Recurrent wheezy chest	10(50.0)	17(21.2)	0.021*
Prematurity	5(25.0)	4(5.0)	0.015*
▪ **Season**			0.026*
Autumn (Oct, Nov, Dec)	11(21.6)	40(78.4)	
Winter (Jan, Feb, Mar)	9(31.0)	20(69.0)	
Spring (Apr)	0	20(100)	

The immunostaining reaction of RSV positive infants revealed positive cytoplasmic reaction in some epithelial cells of nasopharyngeal aspirate which is belong to fusion of the virus with the membrane of individual cells with transmission of viral RNA into the cells. On the other cases the reaction involved the infected cells and the nearby cells with syncytium formation (large multinucleate cells) which give the virus its name. This syncytium is formed due to specific surface glycoprotein (F) on RSV coat which enhances fusion of infected cells and adjacent uninfected cells with merging of their membranes allowing for cell-to-cell transmission of the replicated viral RNA [20].

The study showed that 20 samples (20%) were positive for RSV from the total 100 samples. This was in accordance with previous studies in which the prevalence rate of RSV was 16.5% among infants with ALRTI using immunofleuresence technique [21,22]. On the other hand, by comparing several community-based studies in developing countries, the percentage of RSV isolates was found to be doubled from 23% to 44% by using virus isolation and ELISA [23,24]. While, other studies reported higher prevalence rate of RSV infection with 54% in Brazil during winter season in infants with LRTI with a family history of asthma using rt-PCR [25]. Moreover, RSV infection was reaching up to 56-72% in patients presenting with bronchiolitis or asthmatic bronchiolitis [26]. These different rates of RSV may contribute to different inclusion criteria in these different studies. In general, RT-PCR and nucleic acid detection methods have proven to be more sensitive than DFA for detection of RSV. But the main downfall of these techniques is the high cost especially in developing countries. So, DFA still offers a reliable option for the detection of RSV especially for patients tested within the first 3 days after onset of symptoms [27,22].

By analyzing the risk factors of RSV infection, it was found that; there was statistically significant relationship between age below 6 months and RSV Infection. These results were in agreement with previous researchers who observed that 64% of RSV positive cases were younger than 6 months and 80% of them were in the age group from 2-11months with considering the young age as a risk factor for RSV infection [21,28,29,30, 31]. The relation between the RSV and the young age may belong to immature immune system with the small infant's airways within the first months of life [32,33,34,35].

The present study revealed higher percentage of male sex among RSV positive cases. This was compatible with previous studies that definite the ratio of RSV infection in boys to girls was being 2:1 and they regarded male sex as a strong and independent risk factor for RSV related hospitalization [36,37]. The reason seems to be of anatomic nature where boys have shorter and narrower airways which may predispose for bronchial obstruction [36,38].

This study also revealed higher percentage of RSV positive cases within infants with artificial feeding in comparison to who received breast feeding with insignificant relationship between type of feeding and RSV infection the protective effect of breast milk against RSV infection is attributed to the presence of IgA and lactoferrin as well as to the fact that breast milk promotes maturation probably through the influence of prolactin [30]. On the other hand, [31] did not notice a protective effect of breast feeding against RSV infection.

This study showed significant relationship between bronchiolitis and RSV infection with 80% of RSV positive cases were diagnosed clinically as bronchiolitis and 20% were diagnosed clinically as pneumonia. These results were in agreement with Carroll et al, [39] and García et al, [40] who noticed significant increase in RSV infection among bronchiolitis cases. On the contrary, Hussey et al. [41] reported that 62.2% of RSV positive cases were diagnosed clinically as pneumonia as well as 20.6% with bronchiolitis, 8% with laryngotracheobronchitis (LTB) and 9.2% with other respiratory illnesses. Bronchiolitis induced destroying of ciliated epithelial cells with releasing of cytokines and chemokines that attract additional pro-inflammatory cells which are known to be potent bronchoconstrictors such as macrophages, neutrophils, eosinophils, and natural killer cells to the site of infection. This was associated with increase secretion production, decreased secretion clearance, ineffective surfactant function and release of bronchoconstrictor substances resulting in increased airway resistance, air trapping and wheezing, which are characteristic of severe lower respiratory tract RSV infections [42].

This study also stated insignificant difference between positive and negative cases regarding using antibiotics. 75% of RSV positive cases and 72.5% of negative cases received antibiotics. These findings are pointing to the important of rapid reliable screening test for early detection of RSV infection with avoid unjustified use of antibiotics [43].

The results revealed significant relationship between family history of asthma and RSV infection. Which was previously reported where the percentages of RSV infection was greater among preterm infants with a family history of asthma compared with those who had no such history [44,45,46].

The association of RSV infection with a family history of allergic disorders was discussed controversial. Law et al., (2004) showed no association was found for family history of wheezing or any other allergic disorder with RSV infection [37]. On the other hand**,** Figueras-Aloy et al. [46] noticed the history of wheezing in the family was found to be of statistically significant relationship with RSV infection.

There was statistically significant relationship between winter season and RSV infection. This was in accordance with Fattouh et al., [21] who found that 97% of RSV positive cases presented during the winter season. The prevalence of RSV infection with low temperature may attribute to higher virus activity with more stability in its section. Moreover, cold temperature might drive populations indoor where RSV spreads more readily [47].

The present study showed significant relationship between CHD and RSV infections. Which was in agreement with Navas et al., [48] and MacDonald et al., [49] who observed that infants with cardiac disease at increased risk of RSV infection with significant higher mortality rate compared to children without cardiac disease.

The present study showed a significant relationship between prematurity and RSV infection. This was previously indicated by Lanari [50] and Resch et al. [51] who stated that

prematurity is widely accepted as a major risk category for severe RSV disease in infants which may belong to different factors. According to Wert, [52] prematurity goes along with small immature airways, immature immune system, with incomplete transfer of maternal antibodies. Moreover, the lung development at 30 weeks gestational age (GA) reveals lung volumes being about three times smaller and a surface area being about four times smaller compared to term infants, whereas air space walls have an increased wall thickness by one third of that of term infants.

In the present study, there was a significant relationship between wheezy chest &RSV infections which was in consistent with finding of Sigures, [53] who reported that wheezing is persisting well beyond the period of acute RSV infection. On the contrary, Fattouh et al. [21] noticed that there was insignificant relation between wheezy chest &RSV infection in children above 5 years old age.

In conclusion, Diagnosis of RSV infection by direct immunofluorescence is fast, easy and suitable for screening large numbers. Our results revealed that RSV is an important cause of LRTI in infants especially among young age less than 6 months. RSV infection rate was 20 % among the total 100 infants with ALRTI. There was significant relationship between RSV infection and young age, male sex, winter season and different medical conditions. Such as; family history of asthma, bronchiolitis, recurrent wheezy chest, congenital heart disease and prematurity. So, early rapid diagnosis of RSV infection is recommended as a routine laboratory assay for infants with ALRTI. This can help in starting early anti-viral treatment which can give better prognosis and avoid unnecessary use of antibiotics where most cases were treated as bacterial infections. moreover, further studies are required to accurately define the risk groups who are in need for immunoprophylaxis against RSV as well as Education programs regarding the importance of infection control precautions should be implemented each year prior to the beginning of RSV season.

CONSENT

As per international standard or university standard, patient's written consent has been collected and preserved by the authors.

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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