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COMPARISON OF RAPID ANTIGEN TEST WITH REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION FOR DIAGNOSIS OF RESPIRATORY SYNCYTIAL VIRUS INFECTION IN INFANTS AND YOUNG CHILDREN

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ABSTRACT

Respiratory syncytial virus (RSV) is the most common cause of hospitalization for acute lower respiratory tract infection, specifically bronchiolitis, affecting infants and young children. An association between primary RSV infection and childhood asthma has been suggested. Rapid and accurate diagnosis of RSV infection is crucial for appropriate patient management and infection control. The aim of our study was to compare the diagnostic performance of Binax NOW RSV (BN) as a rapid test for detection of RSV antigen in nasal wash specimens collected from Egyptian infants and young children with the detection of RSV-RNA by reverse transcription polymerase chain reaction (RT-PCR). Furthermore, to study the frequency of RSV infection in Egyptian infants and young children during the winter season 2009/2010 and the effect of certain risk factors including age and gender on the extent and impact of RSV infection. The study included 72 infants and young children, their age ranged from one to thirty six months. They were 43 males and 29 females. They were

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diagnosed and categorized from history, clinical examination and x-ray findings into: acute bronchiolitis (30 cases), bronchopneumonia (22 cases) and 20 asthmatic cases. Twenty healthy age matched children were selected as controls. Nasal wash specimens were collected from cases and controls. These specimens were processed according to the manufacturer's instructions for RSV diagnosis by BN rapid antigen test and by RT-PCR. Out of the 72 tested specimens, 33 (45.83%) gave positive results for RSV–RNA and 29 (40.28 %) gave positive results for RSV antigen by RT-PCR and BN respectively. BN rapid antigen test had a sensitivity of 87.88% and a specificity of 100%. Positive and negative predictive values were 100% and 90.69% respectively. The total agreement was 94.44%. RSV infection was higher among acute bronchiolitis cases (63.33%) compared to bronchopneumonia (36.36%) and asthmatic cases (30%). Infants <1 year old showed the highest rate of RSV infection and male patients were at higher risk than females. In conclusion RSV is a common etiological agent of serious respiratory tract illness (RTI) in infants and young children. RT-PCR is more sensitive than BN in detecting RSV infection. However, BN is a rapid and easy test that can aid in the detection of RSV in pediatrics with symptoms of respiratory infections helping healthcare provider in making patient management decisions at the same office visit. Negative results do not rule out the infection with RSV so negative samples must be tested by another technique like RT-PCR.

Keywords: Respiratory syncytial virus, Nasal wash, RT-PCR, lateral flow, Binax NOW, Infants, Young children.

INTRODUCTION

RSV a negative strand RNA virus is a member of the pneumovirus subfamily of the family *Paramyxoviridae*. It is the most important virus causing lower respiratory tract infections in infants and children. Premature infants, children with chronic lung disease, congenital heart disease and immunocompromised hosts have high risk of a severe disease ***(Simoes and Carbonell-Estrany, 2003; Terletskaia-Ladwig et al., 2005)***. Epidemics of RSV infection

occur every winter in temperate climates, during rainy seasons, or year-round in tropical regions. Nearly half of all infants become infected during their first year of life, and all children have been infected by the age of two ***(Williams et al., 2002; Nair et al., 2010)***. During their first RSV infection, between 25% and 40% of infants will develop bronchiolitis or bronchopneumonia that required hospitalization. Repeated RSV infections can occur ***(Stensballe et al., 2003)***. It may be difficult to differentiate clinically RSV from other seasonal respiratory viruses. ***(Fleming et al., 2003)***. RSV can cause serious nosocomial infections as children are known to shed this highly contagious virus in high titers for up to several weeks ***(Simon et al., 2008)***. Effective approaches to decrease the rates of nosocomial transmission rely on rapid laboratory diagnosis (***Bont, 2009)***. Several lateral flow immunochromatographic assay (LFA) kits designed for rapid RSV antigen detection at point of care are currently available. Binax NOW RSV (Binax, Inc., Inverness Medical, professional diagnostics, Maine, USA) is a FAD approved rapid one-step LFA for the qualitative detection of RSV antigen in nasal wash and nasopharyngeal swab specimens of young children. This study was designed to assess the diagnostic performance of BN for detection of RSV antigen in nasal wash specimens collected from Egyptian infants and young children in comparison with the detection of RSV-RNA by RT-PCR. Furthermore, to study the frequency of RSV infection in Egyptian infants and young children during the winter season 2009/2010 and the effect of certain risk factors including age and gender on the extent and impact of RSV infection in Egyptian children.

SUBJECTS, MATERIAL & METHODS

Subjects:

The study included 72 infants and young children, (age range from one to thirty six months) admitted to the Pediatric Department, Al-Zahraa University hospital in the period from October 2009 to March 2010. Verbal informed consents were obtained from the parents of the children for inclusion in the study and for sample collection. They were 43 males and 29 females. Based on their clinical and

radiological findings, they were categorized into three groups. Group I included 30 cases of acute bronchiolitis. They were presented with breathlessness, cough, wheezy chest with bilateral rhonchi and fever of low to moderate grade with a preceding history of rhinorrhea. Chest X-ray revealed pulmonary hyperinflation with or without atelectasis. Group II included 22 cases of bronchopneumonia presented with moderate to high grade fever, cough, dyspnea. On examination the children were toxic with tachypnea, chest retraction, bilateral rales and occasional rhonchi. The radiological picture was suggestive of consolidation or patchy infiltrates. Group III included 20 asthmatic cases presented with recurrent episodes of wheeze. Twenty healthy age matched children were selected as controls from those attending the hospital for assessment of growth and development.

Specimens: Nasal wash was obtained from each case by using a plastic bulb catheter that is designed to go no further than the tip of the nostril. The patient held in a sitting position with his head tipped back at about 70°. A small amount (2 ml) of normal saline was instilled in each nostril. The content was then aspirated using the bulb catheter by gentle mechanical suction and collected in appropriately labeled collection tube (cryo tube, Nunc). An aliquot of the nasal wash was immediately tested for the presence of RSV antigen by the rapid antigen test (BN). The remainder of each sample was refrigerated at 4°C until transported to our laboratory on ice within few hours. In the laboratory, the specimens were centrifuged at 2000 rpm for 10 min at 4°C. The supernatants of the specimens were frozen and stored at –70°C until further testing by RT-PCR. Also peripheral venous blood samples were collected for routine laboratory investigations including total and differential leukocytic counts and C reactive protein.

1. Rapid Antigen Test

The principle: This test utilizes an antigen capture conjugated antibody and reagents that move laterally by chromatography. A positive result appears as a pink or reddish-purple line at the bottom of the test strip when RSV antigen is present. A pink to purple control line, also near the bottom of the strip, must be present for any result to be valid.

The procedure: The nasal wash specimens were tested immediately after collection by BN following the manufacturer's instructions. Briefly, the device was removed from the pouch just prior to testing and laid flat on work bench. 100 µl of the specimen was added slowly to the sample pad at the top of the test strip. The test device was closed and securely sealed. Test results were read and interpreted 15 minutes after closing the device under good lighting conditions.

RT-PCR

Viral RNA was extracted from 140 µl of each frozen nasal wash specimen using QIAamp viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. Purified RNA was eluted in 50 µl elution buffer.

The viral RNA extract was reverse transcribed using Omniscript Reverse transcription kit (Qiagen) according to the manufacturer's guidelines in 20 µl total reaction mixture contained 10 µl of template RNA, 2 µl of 10X buffer RT, 2 µl dNTPs mix. (contains 5 mM each dNTP), 2 µl (200 µM) of a random primer, 1 µl (10 U) of RNase inhibitor, 1 µl of Omniscript Reverse transcriptase and 2 µl of RNase free water. The mixture was incubated for 60 minutes at 37°C followed by incubation at 93°C for 5 min then chilled on ice.

A primer pair that amplifies 277 bp in the highly conservative region of the nucleocapsid (N) gene of human RSV (between nucleotides 858 -1135) was used in this study as a universal primer for detection of RSV with the following sequences, Forward primer N1: 5'-GGA ACA AGT TGT TGA GGT TTA TGA ATA TGC-3' & Reverse primer N2: 5'-TTC TGC TGT CAA GTC TAG TAC ACT GTA GT-3' ***(Cane and Pringle, 1991)***.

The PCR was performed in 50 µl total volume containing 25 µl PCR master mix (Qiagen), 1µl of each primer, 2 µl cDNA and 21 µl of RNase free water. After an initial denaturation at 95°C for 1.5 min., 35 amplification cycles of denaturation at 93°C for 1.5 min., annealing of the primer at 55°C for 1.5 min., and chain elongation at 72°C for 1.5 min. were performed followed by a final extension for 10 min. at 72°C. Ten µl of the amplified products were analyzed by electrophoresis on 2% agarose gel containing 1µl ethidium bromide (10 mg/mL) in Tris-Acetate EDTA buffer (pH 8.0). Positive bands were identified as compared with 100 bp DNA

molecular size ladder (Invitrogen), using ultraviolet transilluminator. Photographs were taken using gel documentation system.

STATISTICAL ANALYSIS

Data were statistically described in terms of frequencies (number of cases) and relative frequencies (percentage). The sensitivity, specificity, positive predictive value, negative predictive value and total agreement of the rapid antigen test (BN) were calculated relative to RT-PCR as a reference test. Comparison between the study groups was done using Chi-square (X^2) test. A probability value (*P value)* less than 0.05 was considered statistically significant. All statistical calculations were done using Microsoft Excel version 7 (Microsoft Corporation, NY, and USA) and SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 15 for Microsoft Windows.

RESULTS

Demographic features of the study population are illustrated in **Table (1)**. Out of 72 tested specimens, 33 (45.83%) were positive for RSV-RNA by RT-PCR using a primer pair that amplifies a fragment of 277 bp in the highly conservative region of the N gene sequence **(Figure 1)**, while 29 (40.28 %) were positive for RSV antigen by BN test with insignificant difference (P>0.05) **(Table 2)**. All control specimens were negative by both tests. Compared to RT-PCR as a reference test, the sensitivity, specificity, positive predictive value and negative predictive value of BN were 87.88%, 100%, 100% and 90.69% respectively. The overall agreement was 94.44% **(Tables 3 & 4)**. RSV infection was detected in 19/30 (63.33%) acute bronchiolitis cases by both RT-PCR and BN. However, RT-PCR was more sensitive than BN in the detection of RSV infection in bronchopneumonia and asthmatic patients where {8/22 (36.36%) and 6/20 (30%)} versus {6/22 (27.27%) and 4/20 (20%)} were detected by RT-PCR and BN respectively **(Figure 2).** Infants <12 months old showed the highest rate of RSV infection where 18/24 (75%) were positive for RSV-RNA, then the rate of infection decreased to 9/24 (37.5%) in children with age ranged from 12-24 months and 6/24 (25%) in children above 24 months, but the difference was statistical insignificant (P>0.05) **(Table 5)**. The age distribution of

RSV positive cases relative to their clinical diagnosis is presented in **Figure (3)**.The percentage of male children positive for RSV was higher than that of females (48.84% versus 41.38%) but the difference did not reach statistical significance (P>0.05) **(Table 6)**.

Table 1. Demographic Data of RTI Clinically Diagnosed Children and Healthy Control.

Variable	Bronchiolitis n = 30	Bronchopneumonia n = 22	Asthmatic n = 20	Healthy controls n = 20	P Value
Age (mean±SD) months	9.1 ± 3.9	11.5 ± 4.6	14.8 ± 4.8	9.8 ±3.9	>0.05
Sex (♂/♀ratio)	18 /12	13/9	12/8	10/10	>0.05

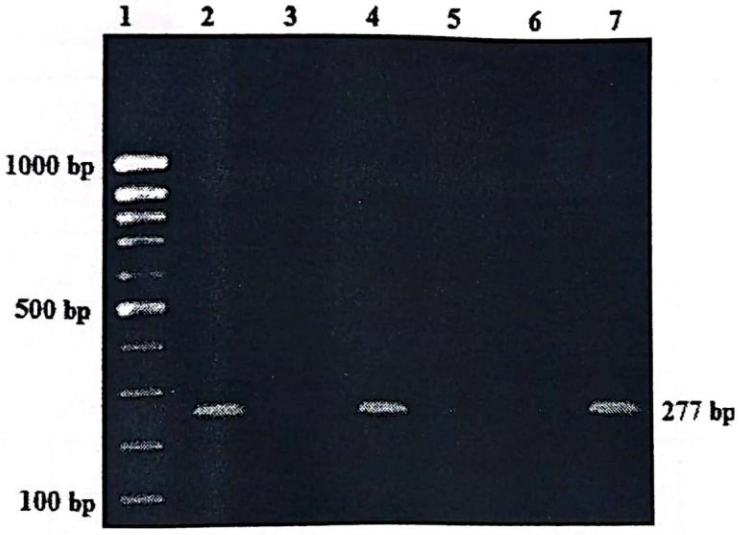

Figure 1. Ethidium bromide stained 2% agarose gel electrophoresis for RSV RT-PCR products: Lane 1 represents 100 bp DNA molecular size ladder (Invitrogen), Lane 3 shows negative control, Lane 2, 4, 5, 6, and 7 show positive amplification of a 277 bp fragment of RSV N gene sequence.

Table 2. Comparison between RT-PCR & Rapid Antigen Test (BN) for RSV Detection.

RT-PCR	Rapid Antigen test (BN)		Total	P value
	Positive	Negative		
Positive	29	4	33	>0.05
Negative	0	39	39	
Total	29	43	72	

Table 3. Concordance between Results of Rapid Antigen Test (BN) and RT-PCR for RSV Detection.

Rapid Antigen test (BN)	RT-PCR	No (%)
Positive	Positive	29 (87.88 %)
Negative	Positive	4 (12.12 %)
Negative	Negative	39 (100 %)
Positive	Negative	0 (0 %)

Table 4. Performance Parameters of Rapid Antigen Test (BN) in Comparison with RT-PCR.

PCR results		Rapid Antigen Test (BN)				
		Sensitivity	Specificity	*PPV	**NPV	Total agreement
Positive	33	29/33 (87.88 %)	29/29 (100 %)	29/29 (100 %)	39/43 (90.69 %)	68/72 (94.44 %)
Negative	39					
Total	72					

*PPV= positive predictive value **NPV= Negative predictive value

Table 5. Frequency of RSV Detection by RT-PCR in Relation to Age.

Age group	Total Number	Positive No (%)	Negative No (%)
1-12 months	24	18 (75 %)	6 (25%)
12-24 months	24	9 (37.5 %)	15 (62.5%)
24-36 months	24	6 (25 %)	18 (75 %)
P value	>0.05		

Table 6. Frequency of RSV Detection by RT-PCR in Relation to Gender.

Gender	Total Number	Positive No (%)	Negative No (%)
Male	43	21 (48.84 %)	22 (51.16 %)
Female	29	12 (41.38 %)	17 (58.62 %)
P value	>0.05		

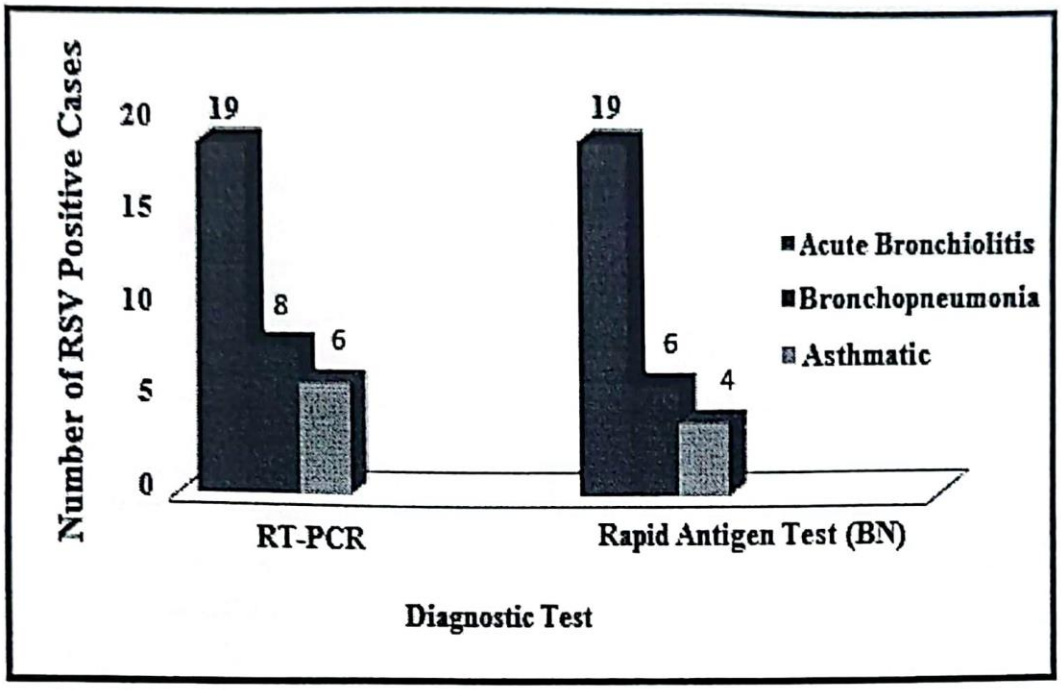

Figure 2. Sensitivities of RT-PCR and Rapid Antigen Test (BN) for RSV Detection Relative to Clinical Diagnosis of RTI.

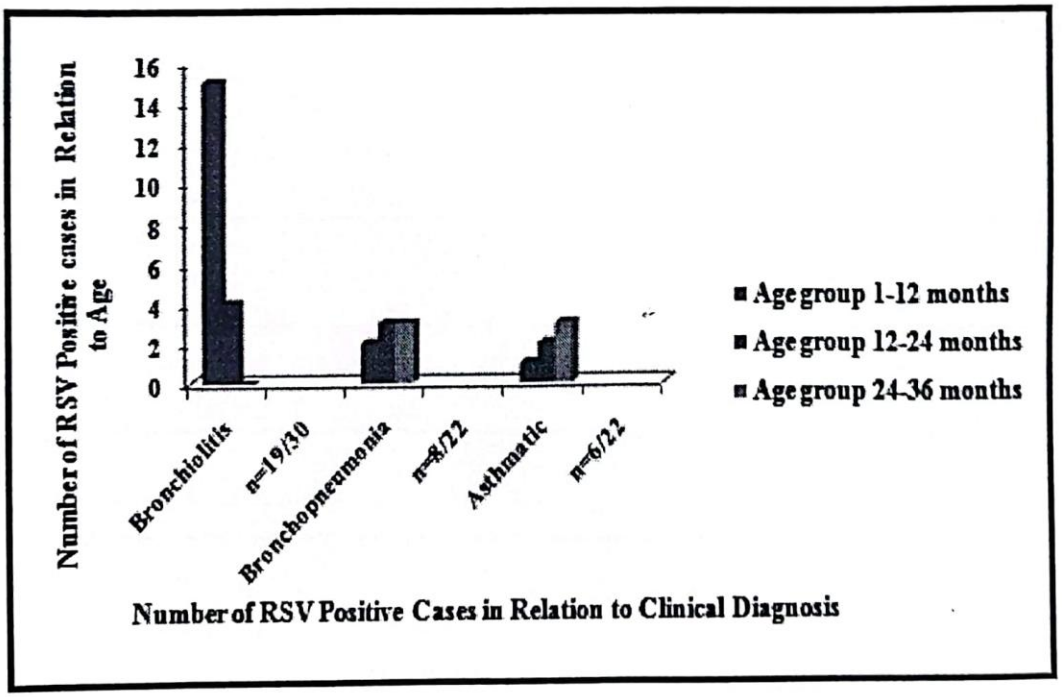

Figure 3. Distribution of RSV Infection Relative to Age and Clinical Diagnosis of RTI.

DISCUSSION

RSV has a worldwide distribution and can cause serious lower respiratory tract illness in infants and young children, particularly under the age of 2 years ***(Hall et al., 2009)***. Furthermore there is mounting interest in the hypothesis that RSV infection in the early childhood is an important risk factor for the subsequent development of recurrent wheezing and asthma ***(Piedimonte et al., 2000)***. RSV results in deaths of over one million children annually as its late detection renders antiviral drugs ineffective. So, it is necessary to diagnose RSV as quickly and efficiently as possible to reduce hospital stays, the cost of hospital care, antimicrobial use and complications ***(Williams et al., 2002)***. The efficiency of diagnosis also allows for proper precautions to be taken to prevent or minimize RSV spread. Previously rapid detection of RSV had been done by direct immunofluorescent assay (DFA) ***(Halstead et al., 1990; Landry & Ferguson, 2000)***. However, it is a subjective test and could give false positive results. Processing and reading of samples by DFA still requires specialized equipment and staff. Virus isolation is considered the 'gold standard' method for RSV diagnosis. However, it requires fully equipped laboratories with skilled professionals and has a long turnaround time. Additionally, RSV tends to be labile and loss of infectivity can occur during transport. Many studies have reported that nucleic acid amplification techniques are more sensitive than viral culture for detecting RSV in clinical samples ***(Falsey et al., 2002; Weinberg et al., 2004; Meerhoff et al., 2008 and Liao et al., 2009)***. This may be explained at least in part by non-viability of viral particles in the specimens that can be detected by RT-PCR while virus isolation requires the presence of viable viral particles to achieve a positive result ***(Jonathan, 2006)***. Several immunoassay antigen detection kits are available, but most require multiple steps in processing ***Hadziyannis et al., 1999)***.

Consequently in our study RT-PCR was used as the gold standard for RSV detection. Samples positive by this method were considered true positives to evaluate BN as one of LFA kits that have been developed for direct qualitative detection of RSV antigen in nasal wash and nasopharyngeal swab

specimens of young children. The results showed that 45.83% of the nasal wash specimens were positive for RSV-RNA by RT-PCR while 40.28 % were positive for RSV antigen by BN with sensitivity and specificity of 87.88% and 100%, respectively. Positive and negative predictive values were 100% and 90.69% respectively and the overall agreement was 94.44%. It was noticed that the sensitivity of BN was higher in children <1 year compared to children >1 year and in children with bronchiolitis compared to children without bronchiolitis as the false negative four cases were > 1 year old and two of them were diagnosed clinically as bronchopneumonia while the others were asthmatic.

Several studies have been done to assess the diagnostic performance of different rapid LFA kits for detection of RSV in respiratory specimens from children in comparison with one or more of the diagnostic methods including DFA, virus isolation in cell culture and RT-PCR.

Ginocchio et al. (2010) compared the diagnostic performance of BN with viral isolation in cell culture and DFA for detection of RSV in nasal wash samples from children below 5 years and they found that the sensitivity and specificity of BN were 75.6% and 100% respectively.

Liao and co-authors (2009) evaluated a real-time RT-PCR assay for detection of RSV-RNA in comparison with viral isolation in cell culture and rapid antigen detection by BN over two successive respiratory virus seasons. The specificity of all methods tested was > or = 99%, and the sensitivity of BN was 82% versus 57% for cell culture and 95% for the real-time RT-PCR.

In a prospective study done by ***Selvarangan and others (2008)*** the performance characteristics of BN assay was compared with RSV isolation in cell culture using 99 fresh nasopharyngeal aspirate specimens from children. Culture negative specimens that tested positive by the antigen test were analyzed by RT-PCR for the presence of RSV–RNA. Specimens positive by culture and/or RT-PCR were considered true positives. The sensitivity, specificity, positive predictive value, and negative predictive value for BN were 90%, 100%, 100%, and 90% respectively.

Cruz et al. (2007) compared BN with viral isolation for detection of RSV in 14,756 pediatric respiratory specimens. The sensitivity and specificity of BN was 81%, and 93.2% respectively. Sensitivity was greatest for neonates (91.1% versus 80.7% for older children [$P < 0.01$]).

Jonathan (2006) evaluated the diagnostic performance of BN RSV in comparison with viral isolation in cell culture and DFA using 100 specimens consisting of 91 nasopharyngeal aspirates, 53 were from infants <5 months old, 27 from children aged 5 months to 5 years and 11 from older children and adults (age range 13 – 52 years). The other 9 specimens were broncho-alveolar lavages (BAL) from adult patients (age range 19 – 69 years). Sensitivity and specificity of BN, DFA and cell culture were 78.9% and 100%; 100% and 95.1%; 58.9% and 100% respectively. Also, ***Borek et al. (2006)*** compared BN results to those of DFA and/or cell culture in the detection of RSV using nasopharyngeal aspirate and wash samples collected from children (n =110) and adults (n = 66). The sensitivity, specificity, positive predictive value, and negative predictive value of BN were 74% and 100%, 100%, and 90%, respectively. The relatively low sensitivity of BN assay in the previous two studies may be due to low sensitivity of it to detect RSV infection in adult samples included in both studies and also in BAL samples included in one study.

Ohm-Smith et al. (2004) compared BN and DFA with viral isolation in cell culture for detection of RSV in fresh nasal or nasopharyngeal aspirates and washes collected from both children and adults. The overall sensitivity and specificity values found for DFA, BN, were 93% and 97%; 89% and 100% respectively. However, when results were analyzed as those from children and those from adults, the sensitivities of DFA, BN, were 93 and 94 respectively in children but in adults DFA was the only rapid test adequate for detection of RSV (sensitivity of 100% compared to 0% for BN). They concluded that the BN assay is sensitive, specific and the easiest to perform for detecting RSV in children but it is insensitive for detecting RSV in specimens from adults.

Mackie et al. (2004) studied the use of BN as a point of care test for the diagnosis of RSV, sensitivity and specificity values of 87% and

94% respectively were obtained when compared to DFA. Also, ***Aldous et al. (2004)*** in a prospective study compared the performance of the rapid antigen test BN with DFA, viral isolation in cell culture and molecular analysis for detection of RSV and they found that the sensitivity, specificity, positive and negative predictive values of the BN were 89.2%, 100%, 100%, and 94.9%, respectively. They concluded that this rapid assay format proved to be cost-effective and simple to use in comparison to DFA and viral culture.

Although, RSV is associated with all RTI in children, it is highly incriminated in lower respiratory tract affection especially acute bronchiolitis. Also, RSV is the predominant pathogen involved in wheezing and asthma among children in preschool age ***(Hall et al., 2009)***. Our results showed that RSV infection was higher among acute bronchiolitis cases (63.33%) compared to those who were suffering from bronchopneumonia (36.36%) and asthmatics (30%). Our study was also designed to evaluate the effect of age and gender as risk factors on the extent and impact of RSV infection in Egyptian infants and young children. The results showed that infants <12 months old have the highest rate of RSV infection as 75% of them were positive and then the rate of infection decrease with increase in age to reach 37.5% in children with age ranged from 12-24 months and 25% in older children.

Regarding the gender as a risk factor, our results delineate that male patients are at higher risk than female patients where 21/43 (48.84%) were positive for RSV in males versus 12/29 (41.38%) in females, that is may be due the short and narrow airways of young males than young females ***(Meissner, 2003)***.

Several studies on Egyptian infants and young children reported higher incidence of RSV infection which can be explained at least in part by the younger age of the studied groups as RSV infection is usually associated with infants. Also different methods of detection may give different results and by the fact that RSV infection rates varied yearly and regionally ***(Panozzo et al., 2007; Nair et al., 2010).***

Moustafa et al. (2010) studied the role of RSV as a cause of pneumonia and bronchopneumonia

in 107 Egyptian children with age ranged from 1-18 months admitted to Cairo University Pediatric hospital during three successive seasons from 2006-2008. RSV was detected in 81/107 (75.70%) nasopharyngeal aspirate samples by both viral isolation in cell culture and detection of RSV-RNA by nested RT-PCR. Other 7 RSV positive cases were detected by nested RT-PCR only rising the total percentage of RSV positivity to 82%. Also, ***Alrefaie et al. (2008)*** studied the frequency of RSV-RNA detection by nested RT-PCR in nasopharyngeal aspirates from 70 children with age ranged from 2-18 months, admitted to Abo-Elrish El Mounira, Pediatric hospital during three successive seasons from 2005 to 2007 with pneumonia and bronchopneumonia. RSV-RNA was detected in 60/70 (86%) nasopharyngeal aspirates samples. 70% of RSV positive samples were from infants younger than 6 months then the incidence decreases with increase in age to reach 26.66 in infants with age ranged from of 6-12months and 3.33% in children above 12 months of age.

Elfky et al. (2002) studied the role of RSV as a cause of acute bronchiolitis in 30 Egyptian infants and young children with age ranged from 0-24 months presented to the emergency room of Cairo University Pediatric hospital during November 2001. Also, 10 healthy age matched children were included as controls. RSV antigen was detected in 25/30 (83.33%) nasopharyngeal aspirate specimens using Abbott TEST PACK RSV rapid enzyme immunoassay. 86.6% of RSV positive cases were < one year old with an evident predominance in males (76.6%).

Gomaa et al. (2002) in a comparative study between enzyme linked immunosorbent assay (ELISA) and immunofluorescent (IF) for detection of antibody against RSV in serum samples collected from 60 infants and young children (49 were suffering from acute bronchiolitis and 11 from pneumonia), found that 47/60 (78.3%) were positive for anti-RSV by ELISA while 39/60 (65%) were positive by IF. They concluded that RSV is a very prevalent pathogen in bronchiolitis and pneumonia of infancy and early childhood in Egypt.

Abushady et al. (1994) in a study conducted on 30 infants and young children admitted to Al Zahraa University hospital with either bronchopneumonia (10 cases), acute bronchiolitis (13 cases) or

asthmatic bronchitis (7 cases), they reported that RSV was detected in 12/30 (40%) nasopharyngeal wash specimens by cell culture and indirect immune-enzyme staining. However, Dot ELISA detected RSV in 14/30 (46.7%). They concluded that Dot ELISA is a very effective technique for diagnosis of RSV infection.

CONCLUSION

RT-PCR is more sensitive than the BN for detection of RSV infection. However, BN is a rapid one-step, and easy test demonstrating acceptable sensitivity and specificity and can aid in the detection of RSV in pediatric patients with symptoms helping healthcare provider in making patient management decisions in the same office visit and preventing nosocomial spread. Negative results do not rule out RSV infection, so negative samples must be tested by another technique like RT-PCR. Furthermore the assay results must be read at exactly 15 min, which decreases flexibility in performing the test. Our results also, give information about the incidence of RSV infection among infants and young children. It is necessary to build RSV surveillance in our country as it's an important pathogen with social and economical impact especially in early childhood

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