



Journal of Medical Sciences

ISSN 1682-4474

science
alert

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JMS (ISSN 1682-4474) is an International, peer-reviewed scientific journal that publishes original article in experimental & clinical medicine and related disciplines such as molecular biology, biochemistry, genetics, biophysics, bio-and medical technology. JMS is issued six times per year on paper and in electronic format.

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J. Med. Sci., 6 (5): 793-799
September-October, 2006

Pneumoslides-M Technique for Rapid Detection of Atypical Pathogens in Critically ILL Children with Lower Respiratory Tract Infections

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Progress in combating atypical pathogens is hampered by the lack of rapid and standardized diagnostic methods. Pneumoslides-M (Indirect immunofluorescence technique for IgM detection) was tested to validate its use as a rapid screening method for detection of atypical pathogens causing severe LRTI in Pediatric Intensive Care Unit (PICU) and to determine its sensitivity and specificity in the diagnosis of Respiratory Syncytial Virus (RSV) and *Mycoplasma pneumoniae* (M. pn.). Sixty children (mean age 3.92±2.74) with LRTI were recruited from PICU, Pediatric Hospital, Ain Shams University. A nasopharyngeal aspirate was collected for viral and M. pn. cultures, whereas a serum sample was taken for Pneumoslides-M and PCR for RSV and M. pn. detection. Pneumoslides-M test detected viruses in 25% of patients, whereas viral culture detected them in 16.7%. RSV was detected by Pneumoslides-M in 11.67% in comparison to 13.3% by RT-PCR. Sensitivity and specificity for detection of RSV was 75 and 98.1%, respectively. M.pn. was recorded by Pneumoslides-M test in 21.67%, 25% by RT-PCR and 3.3% by M. pn. culture. Sensitivity and specificity were 78 and 95%, respectively. Eight patients with severer LRTI showed evidence of mixed infection by Pneumoslides-M test. No characteristic symptom, sign, laboratory, or radiological findings could clearly identify the etiologic agent responsible for LRTI. Pneumoslides-M test was found to be a reasonably sensitive, highly specific, easy, rapid and cost-effective technique for detection of atypical pathogens. We recommend its use in PICU to ensure prompt initiation of the specific antibiotic or antiviral therapy.

Key words: Pneumoslides-M, RSV, *Mycoplasma pneumoniae*, lower respiratory tract infection

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INTRODUCTION

Acute respiratory tract infections (ARTIs), are one of the major causes of morbidity and mortality in young children throughout the world especially in developing countries (Hijazi *et al.*, 1996). Atypical pathogens are important causes of LRTI causing mild to life threatening illness. The most common atypical bacteriae are *Mycoplasma pneumoniae* and *Chlamydia pneumoniae*, viruses such as Respiratory Syncytial Virus (RSV), influenza viruses and adenovirus (Tan, 1999). They commonly occur as co pathogens in mixed infectious pneumonia which is associated with particularly high mortality (Gleason, 2002). The prevalence of each pathogen varies from country to country and could be due to differences in the geographic areas or due to different diagnostic procedures used in different studies (Maitreyi *et al.*, 2000).

Despite the progress made in the diagnosis of LRTI, it takes a few days to identify the causative microorganisms in the blood or sputum samples (Bansal *et al.*, 2004). Cell cultures for viral and atypical bacterial isolation are usually sensitive and detect a broad spectrum of organisms. The time to results may be as long as 14 days (Landry and Ferguson, 2000). Recently, PCR technique has been reported as rapid method for detection of atypical pathogens with a sensitivity that may exceeds that of culture (Falguera *et al.*, 2001). However, PCR assays need specialized equipment and reagents are expensive (Syrmis *et al.*, 2004). New rapid immunofluorescent assay respiratory screen reagents for detection of multiple viruses and bacteria have been developed lately (Zavattoni *et al.*, 2003).

The aim of the present study is to validate of Pneumoslides-M test (Indirect immunofluorescence technique) as a rapid screening method for detection of viral and atypical pathogens in patients with severe LRTIs in Pediatric Intensive Care Unit (PICU) and study the sensitivity and specificity of this methodology in the diagnosis of important pathogens such as RSV and M. pn. as causative agents of severe LRTIs in PICU.

MATERIALS AND METHODS

This study was conducted on 60 children recruited from PICU, Pediatric Hospital, Ain Shams University suffering from pneumonia. Thirty five children were males and 25 were females with mean age 3.92 ± 2.74 . Samples were collected during winter season from December till March 2005. Diagnosis of pneumonia followed World Health Organization Criteria (1994).

Nasopharyngeal aspirate sampling: Nasopharyngeal aspirates (NP) were collected for viral and Mycoplasma cultures. The specimens were transported into sterile vials containing 2 mL cold viral transport medium (Hank's balanced salt solution with 0.5% gelatin) and transported on ice to the laboratory within few hours of collection and maintained at 4°C for 1 to 2 h until processed. To remove mucus from NP aspirates, 5 mL of phosphate buffer saline was added and specimens were pipetted up and down. Samples were centrifuged at 700 xg for 5 min to pellet cells for culture (Landry and Ferguson, 2000).

Blood sampling: Four milliliter of whole venous blood was withdrawn from each child. The samples were centrifuged at 1000 g for 10 min at 4°C. Sera were separated and stored at -20°C until assayed for Pneumoslides-M test and PCR test for Mycoplasma pn. and RSV detection.

Each patient was subjected to:

- Full history taking and thorough clinical examination
- Routine investigations: complete blood picture using Coulter counter T 660, ESR, CRP and chest X-ray (antero-posterior and lateral views).
- Arterial blood gases (pH, PO₂, PCO₂, SaO₂) using blood gas analyzer BEAR5 supplied by Ciba Corning Diagnostics Corp (Medfield, M.A., USA).
- Pneumoslides-M (Vircell-slide, Granada, Spain): Indirect immunofluorescent assay for the simultaneous diagnosis of the main etiological agents of infectious diseases of the respiratory tract. Each slide has 10 wells, each containing one of the following antigens: *Legionella pneumophila* serogroup 1, *Mycoplasma pneumoniae*, *Coxiella burnetii*, *Chlamydia pneumoniae*, Adenovirus, Respiratory syncytial Virus, Influenza A, B, Para influenza serotypes 1, 2 and 3, Cell control. Serum samples were diluted 1:1 with Phosphate Buffered Saline (PBS) then treated with anti-human IgG sorbent. The sorbent treated diluted serum was incubated 90 min at 37°C with the 10 slide wells. The slide washed twice with PBS. A fluorescent secondary IgM antibody was added to the wells and incubated at 37°C for 30 min, then washed twice with PBS. If positive an IgM response (greenish yellow fluorescence) is obtained.
- Non specific viral culture: Vero cell line tissue culture was used for respiratory viruses' isolation from patient's nasopharyngeal aspirate samples. Specific viral cytopathic effect was observed (Wiedbrauk and Johnston, 1993).

- *Mycoplasma pneumoniae* culture: Using Redipac dehydrated culture medium supplied by Mast Diagnostics (Mast Group Ltd., Merseyside UK). Positive results appear as fried egg golden minute colonies 20- 50 μm (Cassell *et al.*, 1994).
- Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) (RSV) we used a conventional RT-PCR. Viral RNA was extracted from 140 μL of serum by using QIAmp Viral RNA Minikit, according to the instructions of the manufacturer. In reverse transcription, the virus specific oligonucleotide primer (antisense) was:

5 [prime]- CTTGACTTTGCTAAGAGCCATCT-3[prime]. For each sample we prepared a mixture:

Buffer RT (5x) 4 μL , dNTP (2 mM) 2 μL , Primer (10 μM) 2 μL , RT enzyme 8U, H_2O 5.5 μL +eluted RNA 5 μL

The PCR 5[prime] - biotinylated primer (sense) was: 5[prime]-GGAACAAGTTGTTGAGGTTTATGAATATGC- 3 [prime]. For each sample we prepared a mixture:

Buffer PCR (10x) 5 μL , dNTP (2 mM) 5 μL , Primer (antisense) 5 μL at 10 μM ,

Primer (sense) 5 μL at 10 μM , Taq polymerase 2.5U, MgCl_2 4 μL , H_2O 24.5 μL +cDNA 5 μL

These two primers previously defined in the human RSV gene by Cane and Pringle (1991), amplify a fragment of 278 bp from subgroup A and B RSV strains.

- Detection of *M. pneumoniae* by RT-PCR: Bacterial DNA was extracted from 300 μL of serum by using Wizard Genomic DNA purification kit (Promega), according to the instructions of the manufacturer. Purified specimen DNA was stored at -70°C until analysis.

The PCR primers used were MPP11 (sense):

5' TGCCATCAACCCGCGCTTAAC and MPP12 (antisense): 5'CCTTTGCAACTGCTATAGTA

The amplified products were detected by gel electrophoresis, these two primers amplify a fragment of 466 bp (Layani-Milon *et al.*, 1999).

Statistical analysis: Standard computer program SPSS for Windows, release 10.0 (SPSS Inc, USA) was used for data entry and analysis. All numeric variables were expressed as mean \pm Standard Deviation (SD). Comparison of different variables in various groups was done using Mann Whitney test. Spearman's correlation test was used for correlating different variables. Chi-square (χ^2) test was used to compare the frequency of qualitative variables among the different groups. For all tests a probability (p) less than 0.05 was considered significant (Daniel, 1995).

RESULTS AND DISCUSSION

Atypical pathogens and viruses constitute a major obstacle in the management of patients with LRTI admitted to PICU. For optimal clinical utility, a diagnostic test for respiratory pathogens must be sensitive, rapid, affordable and able to detect a wide range of pathogens in order to influence clinical decision making (Rocholl *et al.*, 2004).

In the present study, Pneumoslides-M test for viral detection was evaluated and its results were compared to conventional viral cell culture. Pneumoslides-M detected respiratory viruses in 25%, in comparison to conventional viral culture which yielded positive results in only 16.7% (Table 1). However, results of both tests were highly concordant (Table 2). Freymuth *et al.* (1999) showed that culture failed to detect very low levels of viruses. Moreover, Shetty *et al.* (2003) explained that most of conventional viral cultures did not become positive until after the patient had been discharged from the hospital, whereas Pneumoslides-M results were available within 4 h from arrival of sample to the laboratory. Pneumoslides-M test, therefore, appears to be more sensitive and a rapid screening method for detection of viruses among patients with severe respiratory distress who are more likely to benefit from early diagnosis and antiviral therapy (Zambon *et al.*, 2001).

In order to estimate the sensitivity and specificity of Pneumoslides-M test for detecting specific viral pathogen, we compared this test with a gold standard RT-PCR technique for RSV detection (El-Sahrigy *et al.*, 2004). RSV is one of the most common viral respiratory pathogens that leads to acute severe LRTI in children (D'Elia *et al.*, 2005). RT-PCR test detected RSV genome in 13.3%, whereas, 11.67% gave positive RSV results by Pneumoslides-M test (Table 1). Results of both tests were highly concordant (Table 2). Accordingly, the sensitivity and specificity for Pneumoslides-M test using RT-PCR test was 75 and 98.1%, respectively. Landry and Ferguson (2000) reported sensitivity of 89% for IF technique, while Shetty *et al.* (2003) recorded a sensitivity and specificity of 84 and 99%, respectively. The variable sensitivities reported for various IF tests could be explained by the different types of kits used and dependence on the adequacy of the sample (Reina *et al.*, 1996). Nevertheless, they reported that most sensitivities for IF test ranged between 70 to 95%. Although RT-PCR is highly sensitive and specific for detection of respiratory viruses, yet it is not widely available due to its expensive initial cost.

Mycoplasma pneumoniae was recently found to be one of the commonest atypical bacteria in the LRTI in children (Principi and Esposito, 2001). In the present

Table 1: Number and percentage of atypical pathogens detected by Pneumoslides- M, viral culture, M.pn. culture, RSV PCR and M.pn. PCR

Organisms detected	Pneumoslides- M test no. (%)	Culture		PCR	
		Nonspecific viral culture (%)	M. pn. culture (%)	RSV PCR (%)	M .pn. PCR (%)
All viruses	15 (25.00)	10 (16.67)	-	-	-
RSV	7 (11.67)	-	-	8 (13.3)	-
Adenovirus	8 (13.3)	-	-	-	-
<i>Mycoplasma pneumoniae</i>	13 (21.67)	-	2 (3.3)	-	15 (25)
<i>Chlamydia pneumoniae</i>	3 (5.00)	-	-	-	-
Mixed Infection	8 (13.30)	-	-	-	-
RSV+M.pn.	2 (3.30)	-	-	-	-
Adenovirus +M.pn	6 (10.00)	-	-	-	-

Table 2: Concordance between results of Pneumoslides-M, non specific viral culture and RT-PCR (for RSV and *Mycoplasma pneumoniae*)

No of. positive cases	Pneumoslides-M (n = 31)	Non Specific (NS) viral culture (n = 10)	RT-PCR (RSV) (n = 8)	RT-PCR (M. pn.) (n =15)	p-value
+ve Pneumoslides-M(viruses)					
+ve NS viral culture	8	8	-	-	<0.001
+ve Pneumoslides-M(viruses)					
-ve NS viral culture	7	-	-	-	
-ve Pneumoslides-M(viruses)					
+ve NS viral culture	-	2	-	-	
+ve Pneumoslides-M					
+ve RT-PCR(RSV)	6	-	6	-	<0.001
+ve Pneumoslides-M					
-ve RT-PCR(RSV)	1	-	-	-	
-ve Pneumoslides-M					
+ve RT-PCR(RSV)	-	-	2	-	
+ve Pneumoslides-M					
+ve RT-PCR(M. pn.)	11	-	-	11	<0.001
+ve Pneumoslides-M					
-ve RT-PCR(M. pn.)	2	-	-	-	
-ve Pneumoslides-M					
+ve RT-PCR(M. pn.)	-	-	-	4	

p<0.001= Highly significant concordant results

study a high detection rate for *Mycoplasma* (21.67%) was found (Table 1). This result was in accordance with Chaudhry *et al.* (1998) who indicated that M.pn. plays a significant role in the development of LRTI in young children than was previously thought. In addition, in a recent Egyptian study on adults with community acquired pneumonia, M.pn. was detected in 21.4% using Pneumoslides- M test (Rashed *et al.*, 2006). To confirm the results obtained by Pneumoslides-M test in the diagnosis of M.pn. we compared them with those obtained by specific M.pn. culture and PCR test. Culture for M.pn. yielded positive results in 3.3% which were confirmed to be positive by both Pneumoslides-M and PCR tests (Table 1). Accordingly, the sensitivity and specificity of M.pn. culture were 13.3 and 100%, respectively. These results were in agreement with Kraft *et al.* (1998), Tan (1999), Falguera *et al.* (2001) and Nariai (2004) who revealed that diagnostic testing for

atypical bacteria is very difficult because most of these agents can not be easily cultured. They attributed this failure due to extreme fastidiousness of these organisms and /or their presence in low titers. RT-PCR for detection of M. pn. infection yielded positive results in 25% (Table 1). Positive results for M.pn. by Pneumoslides-M showed high concordance with those obtained by RT-PCR (Table 2). Accordingly, sensitivity and specificity for Pneumoslides-M test (considering PCR as gold standard test) were estimated to be 78 and 95%, respectively.

Different theories were placed to explain the failure to detect an antibody response using Pneumoslides-M test in some cases of M.pn. which proved positive by PCR test. Block *et al.* (1995) and Baer *et al.* (2003) attributed this lack in antibody response either to the attenuation of immune response due to severe infection or to individual genetic differences.

Table 3: Relationship between clinical data and diagnosis of RSV and *Mycoplasma pneumoniae*

Clinical and laboratory parameters	RSV			<i>Mycoplasma pneumoniae</i>		
	Positive cases n = 8	Negative cases n = 52	p-value	Positive cases n = 15	Negative cases n = 45	p-value
Age (year) mean±SD	3.56±1.5	3.97±2.89	>0.05	4.2±3.03	3.8±2.67	>0.05
Paroxysmal cough (No, %)	5(62.5%)	21(40.4%)	>0.05	9(60%)	31(69%)	>0.05
History of recurrent wheezing No. %	7(87.5%)	18(34.6%)	<0.05	11(73.3%)	14(31.1%)	<0.05
WBC (cells mm ³) mean±SD	17.72±6.84	15.32±6.19	>0.05	16.42±7.26	15.4±5.98	>0.05
Oxygen saturation (SaO ₂) mean±SD	70.88±10.6	72.28±10.6	>0.05	71.06±11.48	72.43±10.29	>0.05
Radiological Findings (No,%)						
Diffuse interstitial	6(75%)	40(76.9%)	>0.05	5(33.3%)	36(80%)	>0.05
Localized infiltrates and hilar LN	2(25%)	12(23.1)	<0.05	10(66.67%)	9(20%)	<0.05

p<0.05 = significant, p>0.05 = non-significant

The detection rate for infection with *Chlamydia pneumoniae* using Pneumoslide-M test was low in our study group (5%) (Table 1). These patients had past history of asthma and were admitted to pediatric ICU with asthmatic exacerbation and pneumonia. In two studies by Freymuth *et al.* (1999) and Ngeow *et al.* (2005) similar low detection rates for *C. pn.* infections were reported (4.5 and 4.7% respectively). Esposito *et al.* (2000) revealed a significant relationship between *C. pn.* infection and the appearance of wheezing in children, particularly those who gave history of recurrent episodes of wheezing.

Interestingly, 13.3% of patients presenting with severe LRTI had evidence of mixed infection (two organisms) by Pneumoslide-M test (Table 1). RSV and *M. pn.* were both present in 3.3% of patients. The rest (10%) had mixed infection with adenovirus and *M. pn.* It is worth to mention that PCR confirmed the presence of RSV and *M. pn.* in these patients. Mixed infections in LRTI had been described before in many studies by Block *et al.* (1995) and Wubbel *et al.* (1999). Two hypotheses had been suggested. The first suggests that one pathogen simply facilitates the penetration of the other. The second hypothesis states that both organisms act independently to cause respiratory tract infection. Dual infections can increase the severity of LRTI (Maitreyi *et al.*, 2000).

Many investigators tried to link specific clinical, laboratory and radiological features to the etiological diagnosis of LRTIs. Age is one of the factors that might aid in indicating the etiology of pneumonia. A decrease in the mean value of age was noticed in positive RSV patients, whereas an increase in age was noticed in *M. pn.* positive cases (Table 3). Mc Cracken (2000) confirmed that younger children were more prone to RSV infection owing to the small caliber of their airways. However, *M. pn.* and *C. pn.* infections were more common in children older than 5 years of age; younger children appear to have frequent mild or sub clinical infections (Nelson, 2002).

On admission to PICU, no characteristic symptoms or physical findings could clearly identify the etiologic agent responsible for LRTI in this study cohort. This was in

agreement with Marrie *et al.* (2003) and D'Elia *et al.* (2005) who observed that different etiologic causes for pneumonia could not be distinguished on clinical basis alone. It was observed in this study that 60% of patients who proved to be *M. pn.* positive experienced paroxysmal cough which was in agreement with Iwanczak *et al.* (2001). We also noted that *M. pn.* and *C. pn.* infections were detected more often in patients who gave a history of recurrent wheezing, in accordance with previous study by Baer *et al.* (2003).

The present study showed no significant difference in the mean percentages of white blood cell counts and oxygen saturation (SaO₂) between positive cases with atypical pathogens and viruses and those who yielded negative results (Table 3). This was in accordance with Gleason (2002) who reported the failure of routine laboratory methods to differentiate between typical and atypical organisms.

The diagnosis of pneumonia was verified radiologically in all studied cases. A wide range of

abnormalities was detected. The most characteristic radiological features in children with RSV pneumonia were diffuse symmetrical, interstitial inflammatory changes of the lungs (75%). Localized infiltrates and hilar lymphadenopathy were more prominent in *M. pn.* infection (66.67%) (Table 3). However, there was no significant difference in radiological findings between patients with positive results for RSV or *M. pn.* versus those with negative ones. Esposito *et al.* (2000), Iwanczak *et al.* (2001) and Marrie *et al.* (2003) confirmed that radiological findings had a limited capacity to differentiate between various etiologic agents.

In conclusion, the present study highlights the usefulness of Pneumoslide-M as a multiple panel test for rapid detection of several viruses and atypical bacteria. Sensitivity and specificity of Pneumoslide-M for RSV detection was 75 and 98.1%, respectively, whereas they were 78 and 95% for *M. pn.* detection, respectively. Therefore, it was found to be a reasonably sensitive, highly specific, easy, rapid and cost effective in

comparison to PCR technique. It is recommended to use this test in PICU to ensure prompt initiation of the specific antibiotic or antiviral treatment.

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