Molecular Diagnosis of *Mycoplasma pneumoniae* in Children with Atypical Pneumonia: Comparison with the Standard Culture Procedure.

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*Mycoplasma pneumoniae* causes a variety of respiratory tract infections in young children and adults. The standard laboratory methods for its diagnosis are culture and serology, but this agent being fastidious and growing slowly limits the usefulness of culture for routine purposes. Polymerase chain reaction (PCR) technique has recently been used as a rapid method for diagnosis of *M. pneumoniae* infection. In this study we compared culture on selective media as a standard method and direct detection of *M. pneumoniae* DNA in clinical specimens by PCR. Throat specimens from 40 children suffering from atypical pneumonia and 20 apparently healthy children were studied. A significant difference was found between atypical pneumonia cases and controls as regard total leucocytic count and erythrocyte sedimentation rate. Eight cases (17.5%) were culture positive, while 12 (27.5%) were PCR positive. All culture positive cases were PCR positive, PCR sensitivity was 100%, specificity 92% and accuracy 93.3%. In conclusion, PCR is a sensitive and rapid method for diagnosis of *M. pneumoniae* infection, while culture on selective media although being specific but time consuming. The high sensitivity of the test allows early initiation of proper antibiotic therapy to decrease morbidity of the disease.

Community acquired pneumonia remains a common and serious infection. Atypical pneumonia is a term applied to lower respiratory tract infection that are characterized by signs and symptoms of lobar consolidation. This description can apply to diseases caused by a variety of bacterial, viral and even protozoan pathogens (Hindigeh and Carroll, 2000).

*Mycoplasma pneumoniae* and Chlamydia pneumoniae play a more significant role as causes of lower respiratory tract infections in childhood. In particular the incidence of infections caused by these pathogens is high in children aged less than 5 years, the infections themselves seem to be a possible cause of wheezing and may present a more complicated course when not treated with adequate antimicrobial agents (Principi and Esposito, 2002).

In temperate climates, *Mycoplasma pneumonia* is a common respiratory pathogen causing pneumonia and is determined to be the cause of encephalitis (Bitnun et al., 2001). Most children presenting with pneumonia in the industrialized world will have a viral or atypical organism. The clinical features of these atypical pneumonias may be indistinguishable from bacterial pneumonia (Smyth, 2002).

Culture of the throat swab or sputum on special media may demonstrate *Mycoplasma pneumonia*, but growth is detected after 1 week and may reach up to 3 weeks or even 6 weeks (Talkington et al., 1998).

To identify pathogens responsible for acute severe lower respiratory tract infection in children by non-invasive methods, new diagnostic techniques such as polymerase chain reaction (PCR) may help in diagnosis and choice of treatment (Kabra et al., 2003). Recently, PCR becomes an optimal method for rapid detection of *Mycoplasma pneumoniae* in clinical specimens. It has
several advantages over isolation as it is rapid, highly sensitive and specific for the identification of *Mycoplasma pneumoniae* and is helpful for the detection and monitoring of the outbreaks (Waring et al., 2001).

The objective of this work was to develop rapid diagnostic procedure for *Mycoplasma pneumoniae* by detection of its DNA and compare it with the standard culture procedure hoping that rapid diagnosis may help to decrease morbidity and mortality caused by this infection.

**Subjects and methods**

This study was carried out on 60 children classified into 2 groups:

Group I: 40 children. Their ages ranged from 5-12 years. They presented with non productive cough and signs of upper and lower respiratory tract infection for at least 3 days.

Group II: 20 apparently healthy children of same age group served as control.

All studied groups were subjected to the following investigations:

1) Full clinical history, clinical examination and x-ray for the chest.
2) Complete blood picture using Sysmex SF-3000 (TOA Medical Electronico CO-LTD, KOBE, Japan) and Erythrocyte sedimentation rate.
3) Liver function tests: Using Dimension AR (Dade Behring Inc, USA).
4) Throat swab examination:

**Specimens:**

Throat specimens were obtained by a cotton-tipped sterile throat swabs from the space between the palatine arches. Two throat swabs from each patient were obtained.

Throat swabs were placed into 3 ml of transport media formed of:

- PPLO broth (from Difco lot n. 0055003)
- Yeast extract (10%)
- Unheated horse serum (20%)
- Glucose (0.5%)
- Phenol red (0.002%)
- Penicillin (1.000 u/ml)

One of the throat swabs was kept on deep freeze at -70°C for PCR technique while the other swab was cultured within 2 hours from collection.

**Isolation of Mycoplasma pneumoniae:**

Mycoplasma selective supplement P (Oxoid) vials were used. Each vial contains horse serum (20 ml) yeast extract (10 ml), Thallous acetate (25 mg) and pencillin (20,000 units). One vial was make sufficient to make 80 ml of basal medium. Aseptically, 20 ml of sterile distilled water was added to one vial, mixed gently to dissolve. The vial content was added to 80 ml sterile Mycoplasma agar (CM 401), cooled to 50°C, mixed well than poured in sterile petri dishes (Colle et al., 1990). Cultures were incubated aerobically at 37°C from 3 days up to 6 weeks.

**Isolation of other mixed pathogens:**

Specimens were cultured on blood agar, Maconeky agar (aerobically at 37°C) and chocolate agar (in 5% CO₂ at 37°C) (Mackie and McCartney, 1989). The isolated colonies were subjected to colonial morphology, gram stain and biochemical reaction examination.

**Identification of Mycoplasma pneumoniae:**

Colonies of Mycoplasma pneumoniae grow on the selective media as fried egg appearance with darking at the center (McCartney et al., 1996) (Fig.1). Colonies were stained by Diene’s stain as follows:

Clean cover slips were smeared with the stain and allowed to dry. A block of agar with the suspected colonies was cut with a sterile scaple and placed side down on the dry stain. A brass ring was attached to a slide by warming in a flame, followed by pressing the ring against the cover slip with agar block on it and examined microscopically by the low power after 15 min. Colonies of mycoplasma retain the stain, while those of other bacterial colonies lose the colour in half an hour (Mackie and McCartney, 1989).
Detection of Mycoplasma pneumoniae DNA by PCR (Dorigozestma et al., 1999):

1. Throat swabs were preserved in 2 ml of transport media and kept in -70°C until used.

2. Extraction of genomic DNA from specimens: DNA was extracted by first lysing the cells with an anionic detergent in the presence of DNA stabilizer. The DNA stabilizer works by limiting the activity of DNase contained in the cell. Contaminating RNA was removed by treatment with an RNA digesting enzyme while other contaminants such as proteins are removed by salt precipitation. Finally, the genomic DNA was recovered by precipitation with alcohol and dissolved in buffered solution containing DNA stabilizer.

3. Amplification.

(A) Primers:

2 pairs of primers were used as described by Bernet et al. (1989).
Primer 1: 878 μg (5′GAA GCT TAT GGT ACA GGT TGG3′)
Primer 2: 851 μg (5′ATT ACC ATC CTT GTT GTA AGG3′)
This pair was used in 1st step PCR and amplify 144 bp.
Primer 3: 1150 μg (5′ TGA CTG GAA GGA TGT TAA GC3′).
Primer 4: 913 μg (5′ TTG TAA TCG TCT TTA TTT CG3′).
The 2nd pair was used in the 2nd step PCR and amplify 104 bp.

(B) Amplification of DNA:

1- 5 μl of extracted DNA were added to the following mixture:
- Taq polymerase buffer (10x) 5 μl
- MgCl₂ 3 μl
- DNTPS 40 mM (10μM of dATP, dCTP, dGTP and dTTP) 1 μl
- Primer 1 (50 pmol/μl) 1 μl
- Primer 2 (50 pmol/μl) 1 μl
- Sterile distilled water 39 μl
- Total reaction 50 μl

2) Amplification were carried out using thermocycler (Perkin Elemer 2400) as described by Abele-Horn et al., 1998, as follows:

Cycle 1: 95°C for 5 min (once)
Cycle 2: Consists 40 cycles as follows:
a) Denaturation at 95% for 20 sec.
b) Annealing or hybridization of the primers to their complementary sequences on either side of the target sequence at 63°C for 2 min.
c) Extension at 72°C for 1 min during which the polymerase binds and extends a complementary DNA strand from each primer.
Cycle 3: Final extension step for 10 min at 72°C (once).
3) The amplified DNA was kept at 4°C until used for 2nd amplification. Steps were repeated at the same quantities for primers 3, 4.

(C) Detection of amplified DNA product:

10 μl of amplification reaction mixture was analysed by gel electrophoresis in 2% agarose and ethidium bromide staining. The size of the expected amplified DNA band is 104bp (Fig 2).

Statistical analysis:

All data were coded, entered and analysed using EPI info (version 6.1) software computer package (Dean et al., 1994).

Results

Table I shows personal characteristics of the atypical pneumonia cases and controls. There was no significant difference as regards age and sex.

Table (I): Personal characteristics of atypical pneumonia cases and control group.

<table>
<thead>
<tr>
<th></th>
<th>Cases of atypical pneumonia n = 40</th>
<th>Control n = 20</th>
<th>P&lt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>7.1±2.2</td>
<td>8.7±3.5</td>
<td>NS</td>
</tr>
<tr>
<td>Range</td>
<td>(5-12)</td>
<td>(5-14)</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>N %</td>
<td>N %</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>21 %</td>
<td>12 %</td>
<td>NS</td>
</tr>
<tr>
<td>Female</td>
<td>19 %</td>
<td>8 %</td>
<td></td>
</tr>
</tbody>
</table>
Table II shows the frequently of pure *Mycoplasma pneumoniae* infection according to age and gender. There was no significant difference between cases with atypical pneumonia due to *M. pneumoniae* and atypical pneumonia due to other causes as regard age, but a significant differences as regard sex was found.

Table III compares between cases of atypical pneumonia and controls as regard ESR and total leucocytic count. There was a significant difference between the two groups as regard ESR and TLC.

Table (II): Incidence of pure *Mycoplasma pneumoniae* infection according to age and gender.

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Cases of <em>M. pneumoniae</em></th>
<th>Cases of atypical pneumonia due to other causes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
<td>%</td>
</tr>
<tr>
<td>5-6</td>
<td>6</td>
<td>54.5</td>
</tr>
<tr>
<td>&gt;6</td>
<td>5</td>
<td>45.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gender</th>
<th>Cases of <em>M. pneumoniae</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
</tr>
<tr>
<td>Male</td>
<td>9</td>
</tr>
<tr>
<td>Female</td>
<td>2</td>
</tr>
</tbody>
</table>

P<0.05 is significant
NS = Not significant

Table (III): The erythrocytic sedentation rate and TLC results among atypical pneumonia patients and control groups.

<table>
<thead>
<tr>
<th></th>
<th>Cases of atypical pneumonia</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X±SD (range)</td>
<td>X±SD (range)</td>
</tr>
<tr>
<td>ESR</td>
<td>22.05±13.6 (10-64)</td>
<td>8.95±3.1 (3-15)</td>
</tr>
<tr>
<td>TLC</td>
<td>11.1±3.04 (7-18)</td>
<td>6.55±2.3 (4-11)</td>
</tr>
</tbody>
</table>

Table IV shows the frequencies of *Mycoplasma pneumoniae* infection by both culture and PCR techniques. All culture positive cases (17.5%) were positive for PCR. Additional 4 culture negative cases were also PCR positive (27.5%).

Table (IV): The frequency of *Mycoplasma pneumoniae* infection by culture and PCR.

<table>
<thead>
<tr>
<th>Atypical pneumonia</th>
<th>Mycoplasma pneumonia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
</tr>
<tr>
<td>Culture (+ve)</td>
<td>7</td>
</tr>
<tr>
<td>PCR (+ve)</td>
<td>11</td>
</tr>
</tbody>
</table>

Table V shows relation between cases of mixed infection and those with pure *M. pneumoniae* infection. *Streptococcus pneumoniae* was detected in 3 out of the 40 cases (7.5%) and two of them were also *Mycoplasma pneumoniae* positive by PCR technique (6.1%).

Table VI shows validity of PCR in diagnosis of *M. pneumoniae* as compared by culture results. PCR sensitivity was 100%, specificity 92% PV +ve 63.6%, PV –ve 100% and accuracy was 93.3%.
Table (V): Relation between cases with mixed bacterial infection and cases with pure Mycoplasma pneumoniae infection.

<table>
<thead>
<tr>
<th>Culture M. pneumoniae</th>
<th>Total No</th>
<th>Mixed No</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ve</td>
<td>7</td>
<td>1</td>
<td>14.3</td>
</tr>
<tr>
<td>-ve</td>
<td>33</td>
<td>2</td>
<td>6.1</td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>3</td>
<td>7.5</td>
</tr>
</tbody>
</table>

Table (VI): Validity of PCR in diagnosis of *Mycoplasma pneumoniae* as compared by culture.

<table>
<thead>
<tr>
<th>Culture +ve</th>
<th>Total No</th>
<th>MC nemar P</th>
<th>PCR sensitivity</th>
<th>PCR specificity</th>
<th>PV +ve</th>
<th>PV –ve</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ve</td>
<td>11</td>
<td>7</td>
<td>4</td>
<td>2.25 &gt;0.05 NS</td>
<td>100%</td>
<td>92%</td>
</tr>
<tr>
<td>-ve</td>
<td>29</td>
<td>0</td>
<td>29</td>
<td>63.6%</td>
<td>100%</td>
<td>93.3%</td>
</tr>
</tbody>
</table>

Discussion

The temporal association of acute asthma exacerbations in children with viral infections has been known for many years. Recently, the contributions of infections to the pathophysiology of asthma have been expanded beyond disease exacerbation to include disease chronicity and even prevention. Mycoplasma pneumoniae has been correlated with asthma chronicity (Montalbano and Lemanske, 2002). Mycoplasma pneumoniae originally thought to be a virus and called eaton agent, it was found to be a mycoplasma in early 1960. It is the smallest self replicating biologic system (Dwight, 2000). Infections with *M. pneumoniae* was proved to be community acquired, affecting all age groups but was common in infant and preschool children. It occurred all around the year but was common at spring. Recovery from infection usually followed by complications and death occurs in some cases (Madani and Ghamdi, 2001).

This study aimed to evaluate a rapid method for diagnosis of *M. pneumoniae* by detection...
of its DNA in clinical specimens comparing these results with those obtained by culture. Our results showed increased incidence of *M. pneumoniae* infection among male children with no association between age and infection. This finding is nearly similar to that reported by Foy et al. (1979) and Dorigo-Zetsma et al. (1999).

In this study, the most common clinical presentations among cases with pure *M. pneumoniae* infections were cough, malaise, fever, wheeze and coryza and these findings are the same found by Mandani and Ghamdi (2001) and Ahmed et al. (2000). Also patients in this study were characterized by leucocytosis and increase in ESR and this finding is the same of Bjorn and Lebech (2002).

The present study proved that culture positive for *M. pneumoniae* infection were 17.5% of the total atypical pneumonia cases while PCR +ve cases were 27.5%. Results obtained by other authors for detection of *M. pneumoniae*, from throat swab or nasopharyngeal aspirates by culture and PCR, were invariance as follows; Warning et al., (2001) reported culture +ve in 7.8% of cases while PCR +ve in 26%. On the other hand, Ahmed et al., (2000) reported culture positive in 6% and PCR positive in 8%. Dorigo-Zetsmae et al. (1999) reported it as 7% for culture and 8% for PCR, Abele-Horn et al., 1998 reported it as 4% for culture and 8% for PCR while Kessler et al., 1997 proved that 7% of cases were positive by culture and 12% by PCR.

These differences may be due to seasonal variation, difference in media used in culture and different primers used for PCR technique (Ieven et al., 1996). The nested PCR (two step PCR) used in our study allow detection of low concentration of mycoplasma DNA in samples and this increase sensitivity to 10 fold better than single step PCR (Abele-Horn et al., 1998).

In this study, three out of our 40 cases (7.5%) had mixed bacterial infection with *Streptococcus pneumoniae*, two of these cases were positive for *M. pneumoniae* by PCR technique. This finding was in agreement with Pandey et al., (2000) who reported that 10% of their cases are mixed with *Mycoplasma pneumoniae* and other bacterial infections. Thus, rapid diagnostic procedures should be used more frequently to determine the infective aetiology early in the course of illness, as mixed infection in children has important therapeutic implications. In this study, Significant correlation was found between culture and PCR methods in diagnosis of *M. pneumoniae* infection and this finding is the same of Abele Horn et al., 1998 and Dorigo-Zetsma et al., 1999. This means that these patients revealed high respiratory colonization levels and therefore high probability for positive culture. Therefore, the difficulty is not in the detection of mycoplasmas but in the reliable confirmation of a rapid diagnosis which is important for early and adequate antibiotic therapy (Kabra et al., 2003).

The findings in this study that the PCR sensitivity is 100%, specificity 92%, PV +ve 93.6% and PV –ve 100%, is in agreement with Oguz et al., 2002 who reported that PCR sensitivity ranges from 91.6% to 100, specificity from 98.6% to 100%, PV +ve 100% and PV –ve 98.3%. They also reported that PCR must be done during the initial evaluation of the patient for the reliable diagnosis of *M. pneumoniae* which will increase the chance of early and appropriate therapy.

These results are in variance with Ahmed et al., 2000 and Nadala et al., 2001 who reported PCR sensitivity as 80% and 90% and specificity 100% and 98.1% respectively.
They concluded, that low sensitivity of PCR may be due to sample errors resulting in a false negative result because of insufficient amount of DNA.

In conclusion, polymerase chain reaction (PCR) technique is a highly sensitive and fast method for diagnosis of *M. pneumoniae* infection, allowing rapid diagnosis and early initiation of proper antibiotic therapy, hoping for decreasing morbidity of the disease.

References

7. Dean AG, Dean FA, Caulmbier D and Drendel KA (1994): Epi-Info version 6.02 Award processing, Data base and statistics program for public health CDC, USA.


تشخيص الجزئي للميكوبلازما الرئوية في الأطفال المصابين بالالتهاب الرئوي الغير تقليدي مقارنة بطريقة الاستنبات الثابتة

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تسبب الميكوبلازما حوالي 15-40% من الالتهابات الجهاز التنفسي المكتسبة في الأطفال والبالغين والكبار من عدوى الجهاز التنفسي في صغار الأطفال. تراثة الميكوبلازما وقياس كمية الأجسام المضادة في الدم هي الطرق الفيزيائية لتشخيص الميكوبلازما الرئوية ولكن طريقة المزارع شاقة وتأخذ حوالي 5 أسابيع، مما يقل من استخدامها في تشخيص الميكوبلازما الرئوية، وحديثاً تم استخدام تفاعل البلمرة�تسلسل لتشخيص الإصابة بالميكوبلازما الرئوية وهو يتميز بسلاسته ودقة وسرعةه في التشخيص وقد استهدفت هذه الدراسة المقارنة بين طريقة المزارع أو الكشف عن الحمض النووي للميكوبلازما بطريق تفاعل البلمرة المسنسل لتشخيص الالتهاب الرئوي الغير تقليدي في الأطفال.

وقد أجري هذا البحث على 60 حالة مقسمة إلى مجموعتين: مجموعة أطفال أصحاء (20 طفل) ومجموعة تعاني من الالتهاب الرئوي الغير تقليدي (40 طفل) وتتراوح أعمارهم بين 0-12 سنة وقد أجريت لهم الفحوصات الروتينية وكذلك مسحة من الجلد أجرى لها مزينة بالطرق التشخيصية والمختارة لفصل الميكوبلازما كما أجري للفحوصات الدقيقة لفصل الحمض النووي للميكوبلازما. وقد أثبتت هذه الدراسة أن تشخيص الميكوبلازما لا ينصح بالكشف الإكلينيكي لأن أعراضه متقاربة مع أعراض الإلتهاب الرئوي الغير تقليدي المسبب بالفيروسات أو أي أسباب أخرى. كما وجد أن هناك أفرع ذات دلالة إحصائية بين مشاكل الإلتهاب الرئوي الغير تقليدي ومجموعة الأصحاء من حيث سرعة الترسيب وعدد كرات الدم البيضاء.

وقد أثبتت هذه الدراسة أن نسبة الإصابة بالميكوبلازما التي تم تشخيصها بطريقة الاستنبات (المزارع) 17.5% وأن نسبة التشخيص بتفاعل البلمرة المسنسل 27.5% وقد أثبت هذا البحث أن تفاعل البلمرة المسنسل له حساسية عالية وسرعة في التشخيص ولكن ليست له ميزة تحديد النوع بنفس درجة المزارع وأن طريقة المزارع متخص بالخصوصية ولكنها تستغرق وقت طويل.