Application of three uniplex polymerase chain reaction assays for the detection of atypical bacteria in asthmatic patients in Kuwait

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Received 22 April 2012; received in revised form 15 November 2012; accepted 12 December 2012

KEYWORDS
Asthma;
PCR;
Legionella;
Mycoplasma;
Chlamydia;
Kuwait

Abstract
Background: Respiratory infections are known to exacerbate wheezing in many asthmatic patients. We aimed to use molecular methods for the fast detection of Mycoplasma pneumoniae, Chlamydia pneumoniae and Legionella pneumophila in respiratory specimens from asthmatic patients in Kuwait.

Methods: We used uniplex PCR assays to detect the three atypical bacteria in clinical specimens from 235 asthmatic and non-asthmatic patients in Kuwait. A regression analysis was used to identify the risk factors related to the bacterial type. Group comparisons for similarity were conducted and correlation coefficients were calculated using SPSS statistical software.

Results: The detection limits using uniplex PCR for C. pneumoniae, L. pneumophila and M. pneumoniae were approximately 1 pg, 2.4 fg and 12 fg of DNA, respectively. M. pneumoniae PCR positivity was more common in asthmatic patients (15%) than in non-asthmatic subjects (9%) (P < 0.05). A marked difference was observed between patients with acute asthma exacerbation (11%) and patients with chronic (stable) asthma (7%) among Kuwaiti patients; these percentages were 16% for non-Kuwaiti acute asthma patients and 14% for non-Kuwaiti chronic asthma patients (P < 0.201).
There was a weak positive correlation between asthma severity and PCR positivity for *M. pneumoniae*. The PCR results for *C. pneumoniae* and *L. pneumoniae* were found to be statistically insignificant.

Conclusions: The results of this study suggest that infection with *M. pneumoniae* may be related to the exacerbation of asthma symptoms and could possibly be a factor that induces wheezing.

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**Introduction**

Asthma is a prevalent disease with marked effects on quality of life and with economic and societal burden. However, the cause of asthma and its pathophysiology are not completely understood. Recently, the possibility that chronic infection may play a role has been suggested [1,2]. The possible role of atypical bacterial infections in the pathogenesis of asthma is an area of intense discussion. There is an increasing body of literature regarding the association between the atypical bacteria *C. pneumoniae* and *M. pneumoniae* and asthma pathogenesis [3].

Respiratory infections are known to precipitate wheezing in many asthmatic patients and may be involved in the etiopathogenesis of asthma. Recently, studies have investigated the associations of infections with the atypical bacteria *M. pneumoniae*, *C. pneumoniae* and *L. pneumoniae* with both acute exacerbation and chronic asthma [4]. Evidence from human studies links both *M. pneumoniae* and *C. pneumoniae* to new-onset wheezing, exacerbations of prevalent asthma and long-term decreases in lung function, suggesting that these organisms can play an important role in the natural history of asthma [5]. One published case report describes a patient in whom a previous acute mycoplasma respiratory infection led to the initial onset of bronchial asthma [6].

Asthma pathogenesis appears to be the result of a complex combination of genetic and environmental influences. There is evidence that *M. pneumoniae* and *C. pneumoniae* play roles in promoting airway inflammation that could contribute to the onset and clinical course of asthma [6]. *M. pneumoniae* has been associated with hospitalization for the acute exacerbation of bronchial asthma [1,7]. Asthmatic patients frequently develop wheezing after respiratory tract infections [8]. Gil et al. found that *M. pneumoniae* colonizes a greater percentage of patients with asthma than controls and may induce wheezing [8]. Sutherland et al. reported that the atypical bacteria *M. pneumoniae* and *C. pneumoniae* are present in the lower airways of approximately 50% of asthmatics [6]. Additionally, Allegra et al. reported seroconversion to *C. pneumoniae* in asthmatic patients [9].

Asthma is increasingly considered an inflammatory disease and is treated with inhaled and/or systemic corticosteroids. Cases of unusual pneumonias due to *L. pneumonia* associated with high doses of oral steroids have been reported [10].

The etiologic diagnosis of these infections remains difficult. This difficulty is primarily due to the absence of characteristic clinical findings and the lack of available detection methods [11]. *C. pneumoniae*, *M. pneumoniae* and *L. pneumophila* are responsible for community-acquired respiratory tract infections, in particular, atypical pneumonia [8,10,12]. *M. pneumoniae* infections are acute and may require hospitalization, whereas *C. pneumoniae* infections, which are frequently asymptomatic, can be responsible for long-lasting diseases that are more severe in elderly patients [13]. For a specific treatment to be administered, a rapid diagnosis must be obtained. The isolation of *M. pneumoniae* or *C. pneumoniae* requires 6–21 days and is often negative. Rapid laboratory tests such as antigen detection or hybridization are of limited sensitivity, and serology is often inconclusive; thus, gene amplification that permits a rapid and sensitive diagnosis is the method of choice [14,15]. When the presence of different agents is being investigated, a multiplex polymerase chain reaction (PCR) is of interest [16,17]. We have devised a method for the detection of *C. pneumoniae* and *M. pneumoniae* that can be applied to clinical samples collected from in-patients with respiratory symptoms [18,19].

The detection of *M. pneumoniae*, *C. pneumoniae* and *L. pneumophila* in children with respiratory symptoms is usually considered evidence of an acute infection. The current methods for the identification of these agents include cell culture, rapid antigen detection assays, serology (indirectly) and, recently, PCR [20].

Although no rapid method for the microbiological diagnosis of atypical pneumonia (AP) is available
for routine use, the availability of such a test could result in less antibiotic therapy and more precisely tailored antibiotic therapies, resulting in reduced costs, fewer side effects and a reduction in the emergence of resistance [21].

The objective of this study was to determine the prevalence rates of the atypical bacteria *C. pneumoniae, L. pneumophila* and *M. pneumoniae* in asthmatic patients in Kuwait using a uniplex polymerase chain reaction method (uniplex PCR).

**Materials and methods**

**DNA extraction method**

Throat swab samples were obtained using Dacron swabs and were placed in sterile tubes containing 2 ml of Tris-EDTA (TE) solution (2 M Tris HCl + 0.5 M EDTA). These samples were transported quickly to the laboratory for DNA extraction. A simple and sensitive method for DNA extraction similar to the method previously described by us for *M. pneumoniae* and *L. pneumoniae* was used [18,19].

**PCR identification**

We used three separate assays, with one set of primers (uniplex) for each bacterial genus and species and for the detection of bacterial DNA (Table 1).

**Uniplex primers**

To detect *M. pneumoniae*, the P1-178 and P1-331 primers, which amplify a 153 bp fragment (see Table 1) of the cytadhesin gene [22], were used. To detect *C. pneumoniae*, the CpA and CpB primers, which amplify a 463 bp fragment of the conserved 16 sRNA gene [23], were used. To detect *L. pneumophila*, LmipL and LmipR, which amplify a 649 bp fragment of the *mip* (macrophage infectivity potentiator), were used [24].

All uniplex amplification reactions were performed in a volume of 25 μl and contained 5 ml of extracted DNA, 10 mM Tris—HCl (pH 9), 50 mM KCl, 0.01% gelatin, 200 mM deoxynucleotide triphosphates, 1.0 U Taq polymerase and primers and MgCl₂ at the concentrations indicated in Table 1.

**Table 1**

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Primer name</th>
<th>Oligonucleotide</th>
<th>Amplification target</th>
<th>Annealing temperature (°C)</th>
<th>MgCl₂ concentration (mM)</th>
<th>Primer concentration (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mycoplasma pneumonia</em></td>
<td>P1-178</td>
<td>5'-CAATGGCACTACGGCGCC-3'</td>
<td>Cytadhesin gene</td>
<td>55</td>
<td>1.5</td>
<td>0.65 μM</td>
</tr>
<tr>
<td><em>Chlamydia pneumonia</em></td>
<td>P1-331</td>
<td>5'-TGCCAGTGAAATACAGCGC-3'</td>
<td>16sRNA gene</td>
<td>55</td>
<td>1.0 μM</td>
<td>1.0 μM</td>
</tr>
<tr>
<td><em>Legionella pneumophila</em></td>
<td>CpA</td>
<td>5'-ATTATAGGAAAGGCGG-3'</td>
<td>16sRNA gene</td>
<td>55</td>
<td>0.5 μM</td>
<td>0.5 μM</td>
</tr>
<tr>
<td><em>Legionella pneumophila</em></td>
<td>CpB</td>
<td>5'-TCACAGAGAACGTAATTTTT-3'</td>
<td>16sRNA gene</td>
<td>55</td>
<td>0.5 μM</td>
<td>0.5 μM</td>
</tr>
<tr>
<td><em>Legionella pneumophila</em></td>
<td>LmipL</td>
<td>5'-GATTGTATGCTTTAATTTT-3'</td>
<td>16sRNA gene</td>
<td>55</td>
<td>0.5 μM</td>
<td>0.5 μM</td>
</tr>
<tr>
<td><em>Legionella pneumophila</em></td>
<td>LmipR</td>
<td>5'-GATTGTATGCTTTAATTTT-3'</td>
<td>16sRNA gene</td>
<td>55</td>
<td>0.5 μM</td>
<td>0.5 μM</td>
</tr>
</tbody>
</table>
PCR detection of atypical bacteria in asthma patients

PCR amplification using CpnA and CpnB (1 μM each), the PCR cycles were conducted as follows: incubation for 10 min at 95 °C, followed by 40 cycles of 94 °C, 55 °C and 72 °C for 1 min each. A final extension at 72 °C for 10 min was also performed. For *L. pneumophila*, the target DNA sequence was amplified using the LmipL and LmipR primers, both at 0.5 μM. The PCR cycles were conducted as follows: denaturation for 4 min at 94 °C, followed by 35 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 50 °C and extension for 1 min at 72 °C. A final incubation for 10 min at 72 °C was also performed.

The sensitivity of the PCR, measured based on ethidium bromide staining of the amplification products, was estimated with 10-fold dilutions of a standard amount of DNA for each of the three organisms.

The PCR products were detected and analyzed by standard electrophoresis on agarose gels (12 g/l) in 89 mM Tris/89 mM sodium borate/200 mM EDTA buffer, pH 8, and were stained with 0.5 mg/ml ethidium bromide. A 100-bp DNA ladder was used as a molecular marker.

**List of reference bacterial species and strains**

Positive control DNA samples were obtained from the National Culture Type Collection (NCTC) and the American Type Culture Collection (ATCC) and included *L. pneumophila* NCTC 11192, *M. pneumoniae* NCTC 10119 FH and *C. pneumonia* ATCC 1356.

Negative controls (sterile water and DNA from respiratory pathogens such as *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), *Haemophilus influenzae* (ATCC 49766), *E. coli* (ATCC 35150), *Bacillus cereus* (ATCC 11778) and *Enterococcus faecalis* (ATCC 29212)) were also prepared and amplified under conditions similar to those for the clinical specimens described earlier.

**Clinical specimen collection**

The study included 235 clinical sputum specimens collected from 74 acute asthmatic patients, 117 chronic asthmatic patients and 45 non-asthmatic (control) patients at Al-Sabah Hospital, Kuwait, who visited the emergency department or were admitted to the ward. This study was a case–control study. The sample size was determined using an α-level of 5% and a power of 80% (β = 20%) using the Epi Info program.

A total of 190 clinical samples from patients diagnosed with bronchial asthma on the basis of clinical symptoms, signs and pulmonary function were studied.

We studied 190 patients with bronchial asthma (109 males with a mean age of 43.7 years and 81 females with a mean age of 34 years) and 45 subjects without asthma (26 males with a mean age of 45.5 years and 19 females with a mean age of 37 years) who served as controls.

Out of the 190 patients with bronchial asthma, 74 had acute exacerbations of asthma, and 116 had chronic or stable asthma. The diagnosis, drug prescriptions and patient history were obtained from the patient files and used to confirm the asthma cases.

The exclusion criteria were as follows: suspected viral infections; allergic rhinitis or allergic sinusitis; smoking; chronic obstructive pulmonary disease (COPD); a radiological diagnosis of pneumonia, cardiac asthma or pulmonary eosinophilia, including allergic bronchopulmonary aspergillosis (ABPA); antibiotic use within the previous 48 h; and patients requiring intensive care.

Patients were considered to have acute asthma if their daily activities were severely restricted by frequent episodes of moderate to severe asthma symptoms (moderate to severe attacks) that could be controlled only by the regular use of high doses of inhaled corticosteroids.

Chronic asthma included a wide range of clinical findings between mild and severe asthma. Patients with chronic asthma showed chronic mild to moderate symptoms that frequently interfered with daily activities and sleep and required the regular use of managers and anti-inflammatory agents.

**Ethical approval**

The study was approved by the Ethical Committee of the Ministry of Health and the College of Health Sciences, Kuwait. This work was performed in accordance with the code of ethics of the World Medical Association (Declaration of Helsinki for experiments involving humans). Each subject was interviewed, and a signed informed consent was obtained. Age, gender and nationality were recorded.

**Statistical analysis**

Asthmatic patients were compared with regard to the presence or absence of *Mycoplasma*, *Chlamydia* or *Legionella* using the χ² test [25]. In the statistical analysis, the predictor or explanatory variables included asthma type and positive PCR
results for atypical bacteria (Mycoplasma, Chlamydia and Legionella). The patients were segregated into three groups: acute asthmatic, chronic asthmatic and non-asthmatic (control) subjects. These groups were cross-tabulated with the prevalence rates of the three bacterial types to distinguish the severity of the diseases vs the biological effect. Other groups (nationality and gender) were also compared with respect to the bacterial prevalence rates using the \( \chi^2 \) test. All data were statistically analyzed with SPSS version 10.1 (Statistical Package for Social Studies, Chicago, Illinois, USA). Statistical significance was defined as a \( P \)-value less than 0.05 [26].

## Results

To test the specificity of the amplified products, control experiments were performed under the same conditions with DNA from various microorganisms that are likely to be found in human respiratory tract specimens and from other microorganisms that are not respiratory pathogens. The amplification of 463 bp, 153 bp and 649 bp DNA fragments was not observed for genomic DNA from any of these organisms (data not shown). PCR amplification was able to detect the DNA of *C. pneumonia*, *L. pneumophila* and *M. pneumonias*, yielding bands of the correct sizes for each species. The limits of detection for the PCRs were approximately 1 pg, 2.4 fg and 12 pg of DNA, respectively.

The results for *Mycoplasma pneumonias* are presented in Table 2. The PCR results were found to be positive for 10 of the 113 Kuwaiti chronic and acute asthmatic patients (8.8%) and for 1 of the 34 Kuwaiti controls (3%). Five of the 43 patients (11.2%) with acute exacerbation and 5 of the 70 (7.1%) chronic asthmatics were found to be PCR positive. For non-Kuwaiti patients, *Mycoplasma* was detected in 10 of the 113 acute and chronic patients, equivalent to a percentage of 12.9% (Table 2). The difference was not statistically significant (\( P < 0.239 \)) (Table 3).

The *C. pneumonia* PCR was positive for 7% and 4.3% of Kuwaiti acute and chronic asthmatic patients, respectively, with a total of 5.3% for all Kuwaiti asthmatic patients \((n=113)\) (Table 2). In comparison, the Kuwaiti control samples had a positivity rate of 2.9% (Table 2). In non-Kuwaiti patients, *C. pneumoniae* was detected in 6.5% and 5.9% of acute and chronic patients, respectively, with a prevalence of 3.9% for non-Kuwaiti acute and chronic asthmatics when considered together \((n=77)\). In contrast, the prevalence was 0% for non-Kuwaiti controls (Table 2). However, this difference was not statistically significant \( (P < 0.411) \).

*L. pneumophila* was detected in 2.3%, 1.4% and 0% of acute, chronic and control Kuwaiti samples and in 3.2%, 2.8% and 0% of non-Kuwaiti samples, respectively (Table 2), equivalent to an overall prevalence of 1.7% (2 of 113 patients) for Kuwaiti

### Table 2: Summary of PCR results for Kuwaiti asthmatic patients and control and the prevalence of the three atypical bacteria.

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Patients</th>
<th>Acute asthma patients</th>
<th>Chronic asthma patients</th>
<th>Control (KW)</th>
<th>Total (KW)</th>
<th>Control (non-KW)</th>
<th>Total (non-KW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kuwaitis</td>
<td>M</td>
<td>F</td>
<td>Total</td>
<td>M</td>
<td>F</td>
<td>Total</td>
<td>M</td>
</tr>
<tr>
<td>4/30</td>
<td>1/13</td>
<td>11.2%</td>
<td>5–38</td>
<td>0–32</td>
<td>7.1%</td>
<td>1/19</td>
<td>0/15</td>
</tr>
<tr>
<td>Non Kuwaitis</td>
<td>4/16</td>
<td>15%</td>
<td>2/25</td>
<td>3/21</td>
<td>13.9%</td>
<td>1/7</td>
<td>0/4</td>
</tr>
<tr>
<td>Chlamydia pneumonia</td>
<td>M</td>
<td>F</td>
<td>Total</td>
<td>M</td>
<td>F</td>
<td>Total</td>
<td>M</td>
</tr>
<tr>
<td>Kuwaitis</td>
<td>2/30</td>
<td>7%</td>
<td>3/38</td>
<td>0/32</td>
<td>4.3%</td>
<td>1/19</td>
<td>0/15</td>
</tr>
<tr>
<td>Non Kuwaitis</td>
<td>0/16</td>
<td>6.5%</td>
<td>1/25</td>
<td>0/21</td>
<td>5.9%</td>
<td>0/7</td>
<td>0/4</td>
</tr>
<tr>
<td>Legionella pneumonia</td>
<td>M</td>
<td>F</td>
<td>Total</td>
<td>M</td>
<td>F</td>
<td>Total</td>
<td>M</td>
</tr>
<tr>
<td>Kuwaitis</td>
<td>1/30</td>
<td>2.3%</td>
<td>1/38</td>
<td>0/32</td>
<td>1.4%</td>
<td>0/19</td>
<td>0/15</td>
</tr>
<tr>
<td>Non Kuwaitis</td>
<td>1/16</td>
<td>3.2%</td>
<td>1/25</td>
<td>0/21</td>
<td>2.8%</td>
<td>0/19</td>
<td>0/4</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kuwaitis</td>
<td>25.8%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non Kuwaitis</td>
<td>20.9%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Summary of PCR results for Kuwaiti asthmatic patients and control and the prevalence of the three atypical bacteria.
patients and 2.6% (2 of 77 patients) for non-Kuwaiti patients. However, a positive PCR result for *L. pneumophila* was not significantly correlated with asthma severity (P < 0.543) (Table 3).

As shown in Table 3, the comparison of the asthma severity with the types of bacteria detected did not reveal any significant difference between groups (P < 0.239). In regard to the nationality of the patients (Kuwaitis vs non-Kuwaitis), no marked differences were observed between Kuwaitis and non-Kuwaitis with respect to the bacterial type (P < 0.201).

However, a comparison of the prevalence rates of the bacterial species between genders (males vs females) showed different results. *M. pneumoniae* was more prevalent in male asthmatic patients (12.6%) than female asthmatic patients (5%), a difference that was significant (P < 0.048) (Table 3). A regression analysis showed that the best predictor, i.e., the species that had the greatest effect, was *M. pneumoniae*, with an odds ratio of 1.582 (P < 0.208) (Table 3).

### Discussion

In our study, positive PCR results for *M. pneumoniae* were more common for the asthmatic non-Kuwaiti (13%) and Kuwaiti (9%) asthmatic patients than for the non-asthmatic Kuwaiti (3%) and non-Kuwaiti (9.1%) groups. A logistic regression analysis showed that *M. pneumoniae* was the greatest risk factor for asthma (odds ratio 1.7) but had no significant effect on asthma severity (P < 0.17 — CI 0.6—12.4). This result may indicate that acute asthma exacerbations in asthmatics may be more closely related to *Mycoplasma* infection than to infections with the other microorganisms even though the control group had the same odds ratio. These results corroborate the earlier findings that there is a higher rate of *M. pneumoniae* detection among asthmatic patients than among non-asthmatic patients [27].

However, in our study, this marked difference was observed only in Kuwaiti patients with acute asthma exacerbation (5/43 vs 5/70 for acute exacerbation and chronic asthma, respectively). There was a positive percentage distribution between asthma severity and PCR positivity, but no statistical significance was observed. This finding is consistent with the results of a previous study by Kraft et al. [27], who reported a higher detection rate of *M. pneumoniae* in the airways of adults with chronic asthma. Our findings also support these authors’ recommendation to evaluate *M. pneumoniae* infection and, in selected cases, to administer empirical
antibiotic treatment to patients with persistent severe asthma that is poorly controlled despite the use of conventional anti-inflammatory asthma therapy [27].

In contrast to studies that have suggested a link between *C. pneumoniae* infection and the exacerbation of asthma as well as disease initiation [28], we did not find any statistically significant difference in the rate of *C. pneumoniae* detection by PCR among asthmatics and non-asthmatics (4.3% vs 4.8%), and a large difference was observed between patients with acute exacerbation and chronic asthma (7% vs 4.8%). The findings on this issue are not consistent. Although *M. pneumoniae* seems to be more important for asthma pathogenesis and exacerbation than *C. pneumonia* in some studies, the role of *C. pneumoniae* was found to be more significant in other studies. These results are similar to those reported by Martin et al., who found that the throat swabs from 7 of the 55 (12.8%) asthmatic patients were positive for *C. pneumoniae* according to the PCR [29].

Only a few studies have reported an association between asthma and *L. pneumophila*. Lieberman et al. found a higher seroprevalence in asthmatic children than in healthy controls [30]. In our study, a higher rate of *L. pneumophila* PCR positivity was observed in the Kuwaiti asthmatic group (2% vs 0%), but there was no correlation between asthma and PCR positivity for *L. pneumophila* (*P* < 0.54). One of the 43 patients who were PCR positive had acute exacerbation, and 1 had stable asthma. *Legionella* infections, which cause intracellular infections similar to those of potential initiators or exacerbators of asthma such as viruses and *C. pneumoniae*, may also be associated with asthma symptoms. In our study, we did not use the "gold standard" diagnosis method for *Legionella* infections, namely, the culture of the microorganism, so this possibility remains to be investigated.

In summary, we found that infection with *M. pneumoniae* is related to the exacerbation of asthma symptoms and is weakly correlated with the disease severity.

A regression analysis showed that the best predictor, i.e., the bacterial species that has the greatest effect, was *M. pneumoniae*, with an odds ratio of 1.582 (*P* = 0.208). Thus, there was a very small significant effect on the exacerbation of asthma symptoms.

This finding, namely, the correlation of PCR positivity with asthma severity, may be considered as evidence for the causality of this association. The use of antibiotics to treat infections with atypical bacteria could play a role in the management of acute asthma attacks. The possibility of *M. pneumoniae* infection should be considered in cases of acute and severe exacerbations and in difficult-to-treat asthmatics. Empirical antibiotic treatment targeting this pathogen should be administered in select cases only [28]. Although researchers are encouraged by the clinical and experimental success with antibiotic therapy in a subset of asthma patients, long-term antibiotic treatment for asthma may be controversial [2].

Conflict of interest

No conflict of interest to declare.

Acknowledgments

The authors are thankful for the cooperation of the Al-Sabah Hospital ER and Medicine Departments and for the technical help provided by Mr. Mouhamed Esmaeil Qasem (PT) from the Chest Hospital, Kuwait.

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