Original Article

Exhaled breath condensate cytokine level as a diagnostic tool for obstructive sleep apnea syndrome

Yongxia Li a,b,* Virasakdi Chongsuvivatwong b, Alan Geater b, Ao Liu c

a Department of Respiratory Medicine, The 2nd Affiliated Hospital of Kunming Medical University, Kunming, Yunnan, China
b Epidemiology Unit, Faculty of Medicine, Prince of Songkla University, Hat Yai, Songkhla, Thailand
c Department of Respiratory Medicine, Kunming General Hospital of People’s Liberation Army, Kunming, Yunnan, China

Received 1 June 2007; received in revised form 13 November 2007; accepted 13 November 2007
Available online 22 January 2008

Abstract

Background: Relationships between exhaled breath condensate (EBC) and serum cytokines and apnea–hypopnea index (AHI) in patients with excessive daytime sleepiness and loud snoring were evaluated for their potential to predict the severity of obstructive sleep apnea syndrome (OSAS).

Methods: Non-smoking patients with suspected OSAS who had undergone polysomnography (PSG) were selected until 22 non-OSAS, and 22 mild, 22 moderate and 24 severe OSAS cases based on AHI were achieved. Ten healthy smokers served as a smoker control group. Interleukin-6 (IL-6), interleukin-10 (IL-10), tumor necrosis factor-α (TNF-α), and 8-isoprostane were measured in EBC and serum on the morning after PSG and related to OSAS severity using linear discriminant analysis (LDA) and logistic regression (LR).

Results: Biomarker levels, in both EBC and serum, differed significantly across the four groups. Classification by LDA using IL-10 in EBC showed the highest agreement with AHI classification (kappa = 0.88). LR distinguished moderate and severe OSAS from mild OSAS and non-OSAS perfectly using IL-6 in EBC and almost perfectly using IL-10 in EBC (area under the ROC curve = 0.997). The levels of biomarkers among smokers overlapped with mild to severe OSAS patients.

Conclusions: Among non-smoker OSAS suspects, EBC IL-6 and IL-10 have potential to predict severity of OSAS.

Keywords: Obstructive sleep apnea syndrome; Oxidative stress; Inflammation; Cytokines; Exhaled breath condensate; Cost effectiveness

1. Introduction

Obstructive sleep apnea syndrome (OSAS) is a common disorder, affecting 4% of men and 2% of women in middle age [1]. It is associated with increased risk of developing cardiovascular complications, such as hypertension, myocardial infarction, and cerebrovascular accidents [2-11]. Definitive diagnosis of OSAS must depend on polysomnography (PSG), which is available only in tertiary care. The process is time and resource consuming and not readily suitable for use in larger populations.

A number of screening methods have been developed but suffer from poor correlation with OSAS severity or inadequate sensitivity and/or specificity. The Epworth Sleepiness Scale (ESS) could detect daytime sleepiness well but had rather low predictivity for OSAS (area under ROC curve being 0.62) [12]. Severity was found to be related to increased neck circumference but only with a low to moderate correlation coefficient ($r = 0.35$) [13]. Clinical score based on snoring, inter-
ruptured breathing during sleep, daytime sleepiness, obesity and hypertension were shown to differ significantly between patients and the normal groups, but the sample size of that study was small and a significant proportion of OSAS patients had a low score [14]. There is a need to find a new predictive tool that gives acceptable accuracy yet at reasonable cost.

Upper airway obstruction during sleep leads to significant hypoxemia. Consequently, in many OSAS patients cyclical alterations of arterial oxygen saturation are observed with oxygen desaturation developing in response to each event followed by the resumption of oxygen saturation during hyperventilation [15]. This phenomenon has been referred to as hypoxia/reoxygenation and might alter the oxidative balance through the induction of excess reactive oxygen species (ROS), quite like in the sequelae of ischemia/reperfusion injury [16]. In addition, upper airway structure and function are altered in patients with OSAS [17–21]. With recurrent obstruction and reopening of the upper airways during sleep, mucosal congestion in the airway can further increase local inflammation and oxidative stress [18,19,22]. Elevation of levels of serum biomarkers for inflammation and oxidative stress were consistently reported in OSAS patients [22–24]. Analysis of exhaled breath condensate (EBC) has already been used to measure biomarkers of oxidative stress and inflammation in several respiratory diseases [25–30]. Yet there has been no study validating whether the levels of biomarkers in serum and EBC could be used to predict OSAS. If this is the case, the new test could be used widely in field surveys, which can further demonstrate the extent of OSAS in the communities.

Isoprostanes are commonly used as biomarkers of oxidative stress, as they are chemically stable. They are the most reliable biomarkers of lipid peroxidation and oxidative stress and are used as quantitative indices of oxidative stress in vivo [31,32]. Interleukin-6 (IL-6) [33] and tumor necrosis factor-α (TNF-α) [34] have somnogenic potency and are involved in the regulation of sleep, whereas interleukin-10 (IL-10) is a cytokine that has well-documented anti-inflammatory and immunoregulatory activities [35,36]. In this study, we analyzed 8-isoprostane, IL-6, TNF-α, and IL-10 in the EBC and serum of OSAS patients, non-OSAS subjects, and healthy smoker subjects. Realizing that there may be a correlation among these biomarkers, we aimed to identify the best biomarker, either singly or in combination, that can be used to predict severity of OSAS with the best cost-effectiveness ratio.

2. Methods

The study was conducted in the Department of Respiratory Medicine, Kunming General Hospital of People’s Liberation Army (PLA) and the 2nd Affiliated Hospital of Kunming Medical University. Serum and EBC biomarker levels were compared with the apnea/hypopnea index (AHI) as a gold standard obtained from PSG in a cross-sectional study design.

2.1. Study subjects

The study protocol was approved by the Ethics Committee of Prince of Songkla University and of Kunming General Hospital of PLA and of Kunming Medical University. Written informed consent was obtained from all subjects.

Consecutive patients who complained of excessive daytime sleepiness and loud snoring, who had been referred to the sleep laboratory of Kunming General Hospital of the PLA owing to suspicion of OSAS, were used as sources for OSAS patients and non-OSAS controls, depending on the results of their PSG tests. All of these patients were either non-smokers or had completely stopped smoking for more than three months before joining the study. In addition, doctors working at the 2nd Affiliated Hospital of Kunming Medical University who smoked heavily and regularly (>20 cigarettes per day for more than 10 years) without any symptom related to OSAS were used as a smoker control group. All subjects must not have used any form of corticoid steroids or anti-inflammation drug (inhaled, nose, oral, or injectable) for the last 4 weeks. They also must not have had any endocrinological disease, other known cause of sleep disturbance either from disease or from drugs or other interventions, airway inflammation such as rhinitis, asthma, COPD, coronary heart disease with cardiac failure, cerebrovascular disease, diabetes mellitus, psychiatric disorders, or respiratory or systemic infections. A complete physical examination was performed prior to the sleep test, including neurology, cardiopulmonary, and ear, nose and throat examinations. None of the subjects were drinkers or users of any kind of drug.

Height (cm), weight (kg), waist circumference (cm), neck circumference (cm), and neck length (cm) were measured. Body mass index (BMI) was calculated (weight in kg/square of height in meters).

2.2. Pulmonary function test

Pulmonary function tests were performed prior to the sleep test and EBC collection. Forced expiratory volume in 1 s (FEV1), forced vital capacity (FVC), and FEV1/FVC ratio were assessed with a spirometer (MasterScreen PFT, JAEGER, Germany). The highest value of each of these three maneuvers was expressed as a percentage of the predicted normal value. Subjects with FEV1 less than 80% or FEV1/FVC ratio less than 0.7 of predicted value were excluded.
2.3. Polysomnography (PSG)

An appointment was made for each subject to undergo a PSG. All subjects were requested to refrain from any sedative drug, alcohol or coffee for at least one day before the sleep test. All subjects underwent overnight PSG at the Department of Respiratory Medicine, Kunming General Hospital of the PLA. They were monitored continuously for at least seven hours using a 19-channel PSG (Sleep-Screen, JAEGER, Germany). Electroencephalograph (EEG), electrooculography, and chin electromyography recordings were obtained with surface electrodes according to standard methods [37]. Airflow was monitored by a thermistor placed at the nose and at the mouth. Abdominal and chest movements were monitored by respiratory inductive plethysmography. Overnight continuous recordings of oxygen saturation were obtained by finger pulse oximetry. Electrocardiograph and sleep position also were recorded. Snoring was recorded by a microphone placed at the neck. An apnea was defined as complete cessation of airflow for at least 10 s. Hypopnea was defined as reduction in airflow by more than 50% from the baseline for at least 10 s in association with a fall in arterial oxygen saturation of at least 4% and/or an electroencephalographic arousal. AHI was defined as the average number of apneas plus hypopneas per hour of sleep. Subjects were classified into four OSAS groups in accordance with AHI results: 0–4.99 was considered to be control, 5–20.99 mild, 21–39.99 moderate, and 40 or above severe OSAS [38,39]. These cut-points which are used in China were somewhat different from those proposed by the American Academy of Sleep Medicine (AASM) [40], probably because China currently cannot afford to treat a large population with relatively mild OSAS.

Fig. 1. Distribution of biomarker level in EBC and serum by severity of OSAS.
2.4. Exhaled breath condensate

EBC was collected using a condenser (EcoScreen; JAEGER; Wurzburg, Germany) after the completion of sleep monitoring in the morning before 8 a.m. with fasting. The subjects were instructed to clean the oral cavity with water and then breathe through a mouthpiece and a two-way non-rebreathing valve, which also served as a saliva trap. They were asked to breathe at a normal frequency and tidal volume, wearing a nose clip, for a period of 15 min. If the subjects felt saliva in their mouth, they were instructed to swallow it. Condensate in the tube, at least 1–3 ml, was collected as ice at –20 °C, and was immediately transferred to special tubes and stored at –80 °C.

After collection of EBC, venous blood was drawn. The serum was separated by centrifugation at 2000 g for 15 min and stored at the same conditions as EBC until analysed. For healthy smoker subjects their EBC and serum were taken in the same fashion.

2.5. Measurement of biomarkers

An enzyme-linked immunosorbent assay kit (ELISA, Cayman Chemical; Ann Arbor, Michigan, USA) was used to measure concentration of 8-isoprostane, IL-6, TNF-α, and IL-10 in both EBC and serum. The assay was validated directly as previously described [15,41–43]. The detection limits of these assays are 4, 1.5, 2, and 1 pg/ml, respectively.

2.6. Sample size

A sample size of 22 was calculated to test the hypothesis of difference of means among four groups based on a previous study [15]. In this study, we recruited 22 non-OSAS control subjects, 22 mild OSAS, 22 moderate OSAS, 24 severe OSAS, and 10 healthy smoker subjects.

2.7. Statistical analysis

We used R software version 2.6.0 [44] to analyze the data and package Epicale version 2.6.0.2 to draw the dot plots [45]. Means ± SD were main descriptive statistics. ANOVA was used to compare means among groups, and scatter plots and correlation coefficients used to examine relationship between variables. Linear discriminant analysis (LDA) was used to compare the discriminant power of each biomarker and their combination for prediction of OSAS severity. Since the specimen was obtained in the form of serum or EBC, combinations of predictor covariates in the LDA model were exclusively analysed among serum or among EBC tests. One half of the dataset was randomly chosen as a training sample to obtain discriminant equations, which were then applied to the other half for prediction. The individual predicted and the real class of this second half dataset were tabulated for computation of weighted kappa statistic. The weighting scheme for the agreement was 1 if the classes were the same, 0.8 if one was control while the other was mild OSAS or one was moderate OSAS and the other was severe OSAS, 0.2 between mild and moderate OSAS, and 0 otherwise. The whole process of half randomized selection, training and testing of LDA prediction and kappa statistic computation was repeated 1000 times. Mean kappa value from this process was then used for comparison and computation of cost-effectiveness.

As an alternative approach to identify discriminant power of the laboratory test, moderate and severe OSAS were combined into one group, and control and mild into the other. This binary outcome was modeled under logistic regression where the tests or their combination were explanatory variables. Stepwise selection of the model was conducted to obtain the lowest Akaike information criterion (AIC) [46]. Moreover, a receiver operating characteristic (ROC) curve was drawn and the area under the curve (AUC) was computed for each model and further used for cost-effectiveness analysis.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control n = 22</th>
<th>Mild n = 22</th>
<th>Moderate n = 22</th>
<th>Severe n = 24</th>
<th>Smoker n = 10</th>
<th>p value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>14</td>
<td>15</td>
<td>18</td>
<td>17</td>
<td>10</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Age (year)</td>
<td>43 ± 93</td>
<td>48 ± 12</td>
<td>44 ± 13</td>
<td>44 ± 8</td>
<td>41 ± 4</td>
<td>0.05</td>
</tr>
<tr>
<td>BMI (kg/m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>23.3 ± 2.0</td>
<td>25.7 ± 4.2</td>
<td>28.8 ± 5.3</td>
<td>28.67 ± 4.2</td>
<td>22.1 ± 2.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FEV&lt;sub&gt;1&lt;/sub&gt;/FVC (%)</td>
<td>85.0 ± 3.5</td>
<td>83.9 ± 3.5</td>
<td>83.7 ± 2.9</td>
<td>83.4 ± 3.0</td>
<td>88.3 ± 1.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AHI (events/h)</td>
<td>2.9 ± 1.3</td>
<td>14.1 ± 3.5</td>
<td>29.7 ± 5.5</td>
<td>70.1 ± 18.1</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>AHI range</td>
<td>0.4–4.9</td>
<td>6.3–19.6</td>
<td>21.2–38.6</td>
<td>41.2–110.1</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Basic SaO&lt;sub&gt;2&lt;/sub&gt; (%)</td>
<td>93 ± 1.5</td>
<td>91 ± 1.9</td>
<td>91 ± 2.1</td>
<td>88 ± 3.6</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>The lowest SaO&lt;sub&gt;2&lt;/sub&gt; (%)</td>
<td>83 ± 3.5</td>
<td>75 ± 8.3</td>
<td>71 ± 7.7</td>
<td>49 ± 12.1</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Apnea time (s)</td>
<td>16 ± 6.7</td>
<td>29 ± 11.7</td>
<td>46 ± 25</td>
<td>85 ± 35.7</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as means ± SD; AHI, apnea hypopnea index; Apnea time, the longest apnea time during the night; Basic SaO<sub>2</sub>, measurement SaO<sub>2</sub> before sleep.

<sup>a</sup> One-way analysis of variance F-test.
Fig. 2. The correlation matrix and paired scatter plots.

Fig. 3. Distribution of mean kappa value and total cost.
For cost calculation, we assumed a fixed cost for any combination of EBC sample collection of 10 Yuan and for any combination of serum sample collection of 4 Yuan. The variable cost was 60 Yuan for each laboratory (bench work) test. The total cost for each alternative was plotted against the corresponding mean kappa statistic and the AUC value.

3. Results

3.1. Anthropometric data, lung function, and oxygen saturation

Anthropometric data are summarized in Table 1. The age of the five groups was similar. Sex ratios among different OSAS groups were similar, but differed from that of the smoker group, who were all male. BMI increased with increasing severity of OSAS, although BMI of the moderate and severe OSAS groups were similar. The FEV1/FVC ratio was similar in all three OSAS groups. Basic SaO2 was generally low, reflecting chronic hypoxia among these selected suspects. The levels were similar in mild and moderate OSAS groups, but with a sharp drop in the severe OSAS. The trend was even more remarkable for the lowest SaO2 during the night. Finally, the longest apnea time increased with OSAS severity.

3.2. Biomarker measurements

Levels of biomarkers by subject group are summarized in Table 2 and Fig. 1. There were significant differences among means of each biomarker across the groups. Overlapping values were common for 8-isoprostane and TNF-α values in both EBC and serum samples. The level of overlapping was less in serum interleukin-6 and interleukin-10. The least overlapping was in these two biomarkers in EBC. The levels of all biomarkers from the smokers were more or less in the middle zone.

Fig. 2 shows that all biomarkers and AHI were highly correlated with one another. AHI was positively correlated with IL-6, TNF-α, and 8-isoprostane from both EBC and serum, but negatively correlated with IL-10 from both EBC and serum.

3.3. Best discriminations

Fig. 3 gives two-way plots of means of kappa statistics derived from the repeated samples against the total costs. The symbols aligned in vertical columns from left to right represent single and two, three, and four biomarker combinations in serum and in EBC, which determine the cost represented by the X-axis. For single tests, means of kappa statistic ranged from 0.7 (TNF-α in serum) to 0.88 (IL-10 in EBC). For two test combinations the range of kappa values lies in a higher position with the combination of IL-10 + 8-isoprostane in EBC giving a kappa value of 0.92. The lowest combination form was IL-6 + 8-isoprostane in serum giving a kappa value of 0.79. Doing three or more tests further increased the mean kappa value slightly but never to values above 0.95.

Focusing on EBC IL-10, which gives the highest kappa/total cost ratio, the corresponding part of Fig. 1 shows that the level of 39 pg/ml divides moderate-severe OSAS from the mild-control group very well with only one subject from the latter misclassified to the former group. The level of 11 pg/ml of EBC IL-6 perfectly classifies these two groups. Although this biomarker discriminates the two groups more accurately than EBC IL-10, the number of overlapping values between severe and moderate and between mild and control are higher in EBC IL-6 than in EBC IL-10, resulting in a lower kappa value.

Since logistic regression ignores any attempt to classify mild from control and moderate from severe OSAS, the results are therefore in favor of EBC IL-6 which gives an AUC value of 1 and both 100% sensitivity (95% CI 91.9–100%) and 100% specificity (95% CI 92.3–100%). IL-10 was the second best giving an AUC value of 0.997; at the aforementioned cut-point the sensitivity was 100% (95% CI 91.9–100%) and specificity was 97.7% (95% CI 88.5–99.9%). In summary, if the level of IL-10 in EBC was under 39 pg/ml or the EBC

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Biomarker levels in exhaled breath condensate and serum (mean ± SD, pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>n = 22</td>
</tr>
<tr>
<td>EBC IL-6</td>
<td>6.8 ± 1.5</td>
</tr>
<tr>
<td>IL-10</td>
<td>56.8 ± 6.7</td>
</tr>
<tr>
<td>TNF-α</td>
<td>83.7 ± 4.1</td>
</tr>
<tr>
<td>8-iso</td>
<td>12.6 ± 2.2</td>
</tr>
<tr>
<td>Serum IL-6</td>
<td>37.5 ± 10.9</td>
</tr>
<tr>
<td>IL-10</td>
<td>87.3 ± 6.1</td>
</tr>
<tr>
<td>8-iso</td>
<td>17.4 ± 2.2</td>
</tr>
</tbody>
</table>

8-iso, 8-isoprostane.

* One-way analysis of variance F-test.
level of IL-6 was above 11 pg/ml the patient was very likely to have moderate-to-severe OSAS.

Fig. 4 gives the plot of area under the ROC curve from logistic regression against the total cost. The highest symbols from left to right represent the single biomarker and two, three and four biomarker combinations with an AUC of 1. EBC IL-6 alone could achieve an AUC of 1 with the lowest cost. It is therefore the best choice. EBC IL-10 was the second best choice. Both of these interleukins were obviously more cost-effective than other biomarkers and the combinations.

4. Discussion

This study shows that severity of OSAS was strongly correlated with IL-6, TNF-\(\alpha\), and 8-isoprostane in the positive direction and with IL-10 in the negative direction, both in EBC and in serum. The biomarker levels of the smoker group overlapped with those of various severity of OSAS. EBC IL-10 and EBC IL-6 were the two most cost-effective discriminators for OSAS severity. The former was the best by weighted kappa statistic criteria and the latter by logistic regression criteria.

Previous studies have reported various levels of correlation between AHI and various biomarkers \([15,43,47–51]\) with sample sizes ranging from 14 to 30. Our results of correlation coefficients are not much different from theirs, except that we additionally demonstrated a correlation between AHI and IL-6, IL-10, TNF-\(\alpha\), and 8-isoprostane in serum and in EBC. With a larger sample size that covered a wide range of OSAS severity, our study could come up with the discrimination power of these biomarkers.

Our findings are consistent with those of Garey et al. \([52]\), who reported significantly higher levels of TNF-\(\alpha\) and nitrite in the EBC of young healthy smokers than in non-smokers. It is well established that long-term cigarette smoking and COPD are associated with airway inflammation \([53]\), which is reflected in altered levels of cytokines.

A cut-point of AHI at 20 events/h has been shown to be linked with the need for specific therapy such as continuous positive airway pressure (CPAP) \([39]\). The logistic regression model assumes no difference of severity a within each side of the cut-point whereas LDA with kappa statistic allows minor agreement across the 20 events/h AHI demarcation line and maintains small level dissimilarity between control and mild, and moderate and severe. While the LDA-cum-kappa statistical method gives better classification of outcome, the value from the equation is more difficult to use. Thus we suggest that both EBC IL-10 and EBC IL-6 are good discriminators.

It is well known that various biomarkers in EBC are elevated in many other lung conditions. High levels of 8-isoprostane in EBC were found in patients with asthma \([26,41,54]\). This biomarker was reported to reflect the severity of asthma. High levels of IL-6 and 8-isoprostane were found in the EBC of patients with COPD \([27,29,42]\), and they were decreased by antibiotic treatment.
Furthermore, high levels of IL-6 and 8-isoprostane in EBC were also found in patients with stable or exacerbated cystic fibrosis disease [31,28], and IL-6 in EBC was decreased by antibiotic treatment. High levels of 8-isoprostane in EBC were found in patients with ARDS [30]. Once these lung conditions could be excluded from OSAS suspects by routine investigations such as history taking and smoking status, a physical exam, chest X-ray and lung function tests, and EBC biomarkers, especially IL-6 and IL-10, would be valuable in predicting severity of OSAS.

In this study, we excluded central sleep apnea, which is a central nervous system disorder caused by disease or injury involving the brainstem such as stroke, brain tumor, viral brain infection, or chronic respiratory disease. So, whether these biomarkers could be used to predict this type of sleep disorder is unknown and should be determined by further studies.

Our cost-effectiveness analysis suggests that one test of either EBC IL-10 or EBC IL-6 is the most cost-effective predictive tool. The facilities for sample collection and analysis of these tests should be affordable by hospitals in middle income countries, where the more expensive PSG (between 360 Yuan and 780 Yuan in China) is often not available or overwhelmed by huge demand. The strong association with AHI also suggests that the tests may be used for monitoring the progress of OSAS treatment. These biomarker assays may also be used in fieldwork if a portable sample collector is developed. Thus, its role in public health surveys and OSAS control programs, such as screening of drivers and hypertension patients, should not be underestimated.

Among parameters with high correlation coefficients with AHI, EBC IL-6 and EBC IL-10 are the best discriminators by kappa statistic and by logistic regression analysis. Higher discrimination by these two interleukins than by the other biomarkers is not due to a higher correlation coefficient but to the influence of the major cut-point of AHI of 20 events/h, which separates control and mild from moderate and severe OSAS. At this point, there is very little or no overlapping of the biomarker concentration. If this cut-point is moved, the conclusion regarding the best predictor biomarker may be changed.

While we have demonstrated the potential role of these biomarkers as a screening and predictive tool, our sample size is still limited. Different laboratory setups may have different standard values. For example, EBC IL-6 among normal people in different studies has shown a wide range of values (1.6 ± 0.1 pg/ml to 4.9 ± 0.2 pg/m, respectively) [15,55]. The current classification system for OSAS follows the Chinese national standard [39], which was also the same as that used by a previous report [38], but different from others [40]. Similar studies should be repeated in different parts of the world. Both EBC and serum collected in this study were early morning samples, in which the level of biomarker reflecting damage from oxygen surge may still be high. It is worth looking at the change of these biomarker values throughout the day before they can be applied to clinical and field practice.

Acknowledgments

This paper is a part of the first author’s thesis to fulfill the requirement for the Ph.D. degree in the Epidemiology Programme at Prince of Songkla University, Thailand. The authors thank the staff of the Department of Respiratory Medicine, Kunnng General Hospital of People’s Liberation Army, and Dr. Lu Xiaqing for her skillful technical work in performing the laboratory tests.

References
