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Prevalence and rapid detection of *Streptococcus pneumoniae* isolated from lung cancer patients with pneumonia infection.

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Abstract

Background: Lung cancer is a major cause of cancer deaths worldwide. *Streptococcus pneumoniae* was the most common pathogen found among them and has demonstrated an increasing resistance to various antibiotics. Early detection of *S. pneumoniae* and appropriate antibiotic therapy is clinically warranted to improve clinical outcome of the patients and prevent drug resistance. The "RAPIRUN-HS® *S. pneumoniae* HS", an inexpensive, easy and rapid detection kit was used for the detection of *S. pneumoniae*. This study aims to evaluate a rapid novel method of detection of *S. pneumoniae* antigen, to determine the prevalence and antibiotic resistance of *S. pneumoniae* isolated from the respiratory tract of lung cancer patients with pneumonia infection.

Methods: Bronchoalveolar Lavage (BAL) specimens, collected from 183 lung cancer patients with pneumonia were cultured for the presence of bacterial growth and antibiotic resistance was determined by minimum inhibitory concentration assay. The *S. pneumoniae* antigen and DNA were detected using RAPIRUN-HS® and Real-Time PCR respectively.

Results: *S. pneumoniae* (68.1%) was the predominant organism isolated ($p<0.05$) from the samples. Real-Time PCR showed the high sensitivity of 82.6% however, the RAPIRUN-HS® test was highly specific showing specificity rate of 79.1% compared to PCR. Fifty-four (58.7%) isolates were found to be resistance to erythromycin ($p<0.05$). Thirty-two (17.5%) patients died in this study.

Conclusion: *S. pneumoniae* was the predominant organism present in pneumonia patients with lung cancer and RAPIRUN-HS® test is highly specific in detecting *S. pneumoniae*.

Keywords: RAPIRUN-HS®, *S. pneumoniae*, Lung cancer, Pneumonia, Antibiotic resistance.

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Introduction

Lung cancer is the major reason of cancer deaths worldwide. In the year 2012, deaths due to lung cancer were estimated to be 1.6 million [1]. The incidence of lung cancer is growing rapidly in China which results in large socioeconomic burden. In 2011, the reported incidence of lung cancer in China was 48.32 per 100,000 populations [2]. Lung cancer patients often suffer from the frequent infection which not just frustrate the impact of oncological treatment but also affects the overall survival [3,4]. Pneumonia and bronchitis were the most common respiratory tract infection caused in lung cancer patients. These infections are mainly due to the colonization of potentially pathogenic and opportunistic microorganisms in the upper respiratory tract [4-6]. Cancer patients are highly susceptible to severe pneumococcal infections and *Streptococcus pneumoniae* was the most common pathogen found in them [7,8]. The incidence of pneumococcal infection increasingly high, leading to higher morbidity and mortality [9]. *S. pneumoniae* has demonstrated an increasing resistance to various antibiotics [10]. Thus an early detection of *S. pneumoniae* and selecting an appropriate antibiotic therapy is clinically warranted to improve clinical outcome of the patients and prevent drug resistance [9,11]. Fever is the constant and the only indicator of infection [12]. Bacteremia can occur in more than 60% of pneumococcal pneumonia cases [9]. Data regarding the colonization of *S. pneumoniae* in pneumonia patients with lung cancer are scarce. In general, the culture method is considered to be the gold standard techniques however, it requires a longer time to obtain the result. Thus a rapid identification of microorganism that colonizes the respiratory tract in lung cancer patients may impact the decision of perioperative antibiotic treatment and prophylaxis. Molecular methods such as PCR and Real-Time PCR were adopted for the detection of bacteria in blood, however; these tests are expensive and require technically skilled personnel. In addition, the high detection rate of real-time PCR leads to over-diagnosis [13]. The "RAPIRUN-HS® *S. pneumoniae* HS" (Otsuka Pharmaceutical, Tokyo, Japan; RAPIRUN-HS® HS) is an inexpensive, easy and rapid detection kit, developed to

detect *S. pneumoniae* from middle ear fluids and otorrheal and nasopharyngeal secretions since 2011. RAPIRUN-HS® detects the capsular, common cell wall and cell membrane antigens of all serotypes of *S. pneumoniae* [14].

For cancer patients, indices such as pneumonia severity index and CURB-65 used to predict the severity and diagnosis of community-acquired pneumonia cannot be used as in the way it is been used for the general population [15]. Thus, in our study patients were categorized to have pneumonia when they have to infiltrate on chest radiograph and the presence of one or more conditions such as fever (≥ 38°C) or hypothermia (<35°C), dyspnea, pleuritic chest pain, new cough with or without sputum production, and altered breath sounds on auscultation [16]. The objective of this study is to evaluate a rapid novel method of detection of *S. pneumonia* antigen for its clinical utility based on the results obtained by Real Time PCR and conventional culture method using Bronchoalveolar Lavage (BAL) specimens. To determine the prevalence and antibiotic resistance of *S. pneumoniae* isolated from the respiratory tract of lung cancer patients with pneumonia infection.

Patients and Methods

A total of 183 BAL specimens from lung cancer patients who were on chemotherapy and/or radiotherapy diagnosed with pneumonia collected between January 2015 and May 2016 were included in this study. About 2 ml of blood was collected from each patient the serum sample was separated and stored at -20°C until used. The *S. pneumoniae* antigen was detected using "RAPIRUN-HS® *S. pneumoniae* HS" kit (Otsuka Pharmaceutical, Tokyo, Japan; RAPIRUN-HS®) as per manufacturer's instruction. Written informed consent was obtained from all the patients or their legal representatives after describing the nature of the study. The study was approved by the institutional ethical committee. Patients were qualified for the study based on histological evidence of lung cancer. Pneumonia was defined as described by Garcia-Vidal et al. [16].

Bronchoalveolar Lavage (BAL) specimens were collected from patients as describe by Dancewicz et al. [17]. Briefly, using sterile bronchoscope fixed in the lobar bronchus of tumor location, 100 ml of sterile normal saline in fractionated doses was injected and then BAL was removed by suction and collected in a sterile suction device. Specimens were mixed and analysis of bacterial growth was performed. Using a sterile calibrated loop specimens were cultured on 5% sheep blood agar, chocolate agar and MacConkey agar (Biobasic, Canada) at 37°C for 48 h in presence of 5%-10% CO_2. Bacterial isolates were identified using conventional biochemical methods.

Detection of *S. pneumoniae* DNA was performed by Real-time PCR using the *S. pneumoniae*-specific capsular polysaccharide biosynthesis (*cpsA*) gene primers described by Park et al. [18]. Using the REDExtract-N-Amp™ Tissue PCR Kit (Sigma, USA) DNA from BAL specimens were extracted and subjected to real-time PCR using the following primer *cpsA*-348F: 5'-GCTGTTTTAGCAGATAGTGAGATCGA-3' and *cpsA*-415R: 5' -TCCCAGTCGGTGCTGTCA-3'. DNA was amplified with the QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems) using the following cycling parameters: 95 1C for 10 min, followed by 40 cycles of 95 1C for 15 s and 60 1C for 1 min. Amplification data were analysed using the inbuilt intuitive software of the system. Melt-curve analysis was also performed to detect non-specific amplifications. If two of the three triplicate yielded a positive result within the <40-cycle cut-off was considered to be positive. The PCR results were compared with the RAPIRUN-HS®; considering the microbiological analysis of BAL specimen that shows a positive result for the presence of *S. pneumoniae* as the gold standard, sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) were calculated.

Minimum Inhibitory Concentration (MIC) of various antibiotics such as penicillin, amoxicillin, cefotaxime, cefepime, erythromycin, levofloxacin and clindamycin (Sigma-Aldrich, USA) against *S. pneumoniae* was determined. MIC assay at a concentration ranging from 0.03 µg/ml to 128 µg/ml was performed by micro broth dilution method; using Muller-Hinton broth supplemented with 5% lysed horse blood as described by Clinical Laboratory Standard Institute (CLSI) guidelines [19].

Statistical analysis

Data with continuous values were represented as medians and ranges and categorical values as numbers and percentages. A Chi-Square and a student t-test were performed to determine statistical significance and a regression analysis was performed to determine the relationship of two variable using SPSS software package (SPSS, version 13.5; SPSS Inc., Chicago, Illinois). A P value<0.05 was considered to be statistically significant.

Results

All the 183 lung cancer patients who were included in the study were presented with fever or hypothermia and diagnosed to have pneumonia. All patients were in chemotherapy or radiotherapy or a combination of both treatments. Among them 108 (59%) patients were male and 75 (41%) patients were female. Majority of the patients belong to the age group of 51-60 y (64, 35%) followed by 41-50 y (49, 26.8%), 31-40 y (37, 20.2%), 22-30 (24, 13.1%) and 61-73 y (9, 4.9%). Demographic and clinical characteristics of patients were summarized in Table 1.

Among the patients whose BAL aspirations were subjected to microbiological analysis, 135 (73.8%) samples (76 from male and 59 from female) showed significant growth and the remaining 48 (26.2%) patients did not show any presence of growth. There was no significant association between the presence of bacterial colonization with sex, smoking habit and location of the tumor (p>0.05). Of the 135 samples which showed the presence of growth, *S. pneumoniae* (92, 68.1%) was the predominant organism isolated followed by *K.*

pneumoniae (21, 15.6%). The presence of *S. pneumoniae* was found to be significantly higher ($p<0.05$) among the isolates (Table 2). There were 13 episodes of polymicrobial colonization, of which 4 patients were isolated with more than 3 microorganisms. In addition to the presence of *S. pneumoniae*, other organisms present include *P. aeruginosa*, *K. pneumoniae*, *S. aureus* and *E. coli*. Among them, *S. pneumoniae* along with *K. pneumoniae* was the predominant combination found in 5 patients followed by the combination of *S. pneumoniae* and *P. aeruginosa* in 4 patients (Table 3).

Of the 183 samples, 109 samples were found to be positive for PCR and 89 samples were positive for RAPIRUN-HS® test. When compared with culture results, 70 samples which showed the presence of growth for *S. pneumoniae* were also found to be positive for RAPIRUN-HS® test (true positive) and 72 samples were found to be negative for both tests (true negative) (P=0.963). Seventy-six samples which showed the presence of growth for *S. pneumoniae* were found to be positive for PCR (true positive) and 58 samples were found to be negative for both tests (true negative) (P=0.811) (Table 4). Based on the obtained results, Real-Time PCR showed high the sensitivity of 82.6% when compared to RAPIRUN-HS® test (76%). However, the RAPIRUN-HS® test was highly specific showing specificity rate of 79.1% compared to PCR (63.1%) towards the detection *S. pneumoniae* detection. The PPV of RAPIRUN-HS® test (78.7%), was higher than that of PCR (69.7%) (Table 5). The Pearson correlation test showed an excellent correlation ($r^2=0.997$) between the RAPIRUN-HS® and the PCR test (P=0.00).

All the tested isolates were susceptible to penicillin using non-meningeal breakpoint. However, 54 (58.7%) isolates were found to be resistance to erythromycin, 14 (15.2%) isolates were resistance to amoxicillin, 2 (2.2%) isolates were resistance to cefotaxime, 2 (2.2%) isolates were resistance to levofloxacin and 1 (1.1%) isolate was resistance to cefepime (Table 6). The presence of erythromycin resistance was significantly higher ($p<0.05$) among the isolates.

All the 183 patients were treated with empirical antibiotics. The most commonly administered antibiotics were ceftriaxone, amoxicillin, cefepime, and levofloxacin. Twenty-one patients were admitted in intensive care unit due to the severity of disease and 12 patients were supported with mechanical ventilation. With an overall mortality of 17.5%, 32 patients died during the course of treatment. Of the 32 deaths 8 patients died due to the progression of pneumonia leading to severe pneumonitis which includes 3 patients who had a ploy-microbial infection of more than 2 organisms, 16 patients died due to rapid progression of lung cancer and 8 patients died due to multiple therapeutic failures.

Table 1. *Characteristics of patient population.*

Demographic characteristics	No of patients (n=183)
Age-y (Median and range)	57 (22-73)
Male	108 (59%)
Female	75 (41%)
Smoker	121 (66.1%)
Non-smoker	62 (33.9%)
Types of lung cancer (histology)	
Squamous cell carcinoma	57 (31.1%)
Adenocarcinoma	38 (20.8%)
Large cell carcinoma	31 (16.9%)
Small cell carcinoma	25 (13.7%)
Anaplastic small cell carcinoma	19 (10.4%)
Carcinoid	13 (7.1%)
Location of tumor	
Right lung	98 (53.6%)
Left lung	85 (46.4%)

Table 2. *Prevalence of bacterial colonization from BAL specimen.*

Bacterial species	No of isolates (n=135)
S. pneumoniae	92 (68.1%)
K. pneumoniae	21 (15.6%)
P. aeruginosa	15 (11.1%)
Staphylococcus sp.	5 (3.7%)
E. coli	2 (1.5%)

Table 3. *Patterns of mixed culture of patients with polymicrobial infection.*

No of patients	Patterns of mixed culture
1	*S. pneumoniae, S. aureus, E. coli*
2	*S. pneumoniae, S. aureus, P. aeruginosa*
1	*S. pneumoniae, K. pneumonia, P. aeruginosa*
5	*S. pneumoniae, K. pneumoniae*
4	*S. pneumoniae, P. aeruginosa*

Table 4. *Comparison of RAPIRUN-HS® and PCR test results.*

	RAPIRUN-HS®	PCR
True positive	70	76
True negative	72	58
False positive	19	33
False negative	22	16
Total	183	183

Table 5. *Sensitivity and specificity of RAPIRUN-HS® and PCR test.*

	RAPIRUN-HS® (%)	PCR (%)

Sensitivity	76	82.6	Positive predictive value	78.7	69.7
Specificity	79.1	63.7	Negative predictive value	23.4	21.6

Table 6. Antibiotic resistance and MIC of S. pneumoniae isolates.

Antibiotics	No of isolates (n=92)			MIC_{50} (µg/ml)	MIC_{90} (µg/ml)	Range (µg/ml)	*CLSI Breakpoint (µg/ml)		
	S	I	R	S	I		R		
Penicillin	92	0	0	≤ 0.03	1	≤ 0.03-2	≤ 2	4	≥ 8
Amoxicillin	66	12	14	1	16	0.06-64	≤ 2	4	≥ 8
Cefotaxime	85	5	2	0.5	0.5	0.12-16	≤ 1	2	≥ 4
Cefipime	78	13	1	0.5	2	0.06-8	≤ 1	2	≥ 4
Erythromycin	17	21	54	1	64	≤ 0.03->128	≤ 0.25	0.5	≥ 1
Levofloxacin	58	32	2	2	4	0.5-16	≤ 2	4	≥ 8
Clindamycin	75	17	0	0.12	0.5	0.06-0.5	≤ 0.25	0.5	≥ 1

*Non-meningeal breakpoint

Discussion

Despite the availability of newer antimicrobial agents and advanced treatment strategies, respiratory infections are common among cancer patients [20]. Pneumonia is one of the major complications occurring in lung cancer primarily due to the failure of clearance system in peripheral bronchial obstruction sites or stenosis brought about by cancer therapy or the cancer itself [21]. It's a known fact that pneumonia is generally preceded by microbial colonization of lower respiratory airways [22]. With the significantly impaired clearance system, bacterial colonization with *S. pneumoniae* and *Haemophilus influenza* is common [23]. A highly sensitive and specific fiberoscopic bronchoscopy was used to obtain sterile specimens for from the lower respiratory tract. In our study, the BAL specimen collected from the lower part of lung yielded both gram positive and gram negative microorganisms. Our study reported that 73.8% of specimens showed the presence of bacterial growth. The majority of the culture positive specimens grew *S. pneumoniae* (68.1%), which was significantly higher ($p<0.05$) when compared to other isolates. Our result corroborates well with Garcia-Vidal et al. [16] from Spain reported that *S. pneumoniae* were the predominant isolate found among cancer patients. Similarly, a study from Poland reported that *S. pneumoniae* were the most predominant microorganism found among lung cancer patients [17]. In our study, the presence of *S. pneumoniae* was followed by *P. aeruginosa* (15.6%), *K. pneumoniae* (11.1%), *S. aureus* (3.7%) and *E. coli* (1.5%). In contrast, Dias and Sreevidhya, [20] from India reported that *P. aeruginosa* was the predominant isolate which caused pneumonia among the cancer patients followed by *S. aureus* and *K. pneumoniae*. Vento et al. [24] from Italy reported that *P. aeruginosa* and *S. aureus* were the predominant isolates found among cancer patients after chemotherapy, followed by *E. coli*. When compared with our report of *S. pneumoniae* predominance among cancer patients, the contrasting evidence of the above studies alludes that there might be a regional variation in the presence of microbial colonization among cancer patients. In most of the lung cancer patients, infections were caused by a single organism however, there were 13 episodes of poly-microbial infection. The ploy microbial pneumonia was commonly associated with *P. aeruginosa*, and *K. pneumoniae* which increase the morbidity and mortality of the patients. In our study, all 3 patients who had a poly-microbial infection of more than 2 microorganisms died during the course of treatment due to severe infection. The overall mortality in our study was 17.5%.

In the modern medical world, rapid diagnosis and treatment play an important role in life and death. The rapid spread of tumor cells in a lung cancer patient may deteriorate the clinical condition of the patients and make them highly susceptible to infections. *S. pneumoniae* which lead to severe pneumococcal infections is the most common pathogen found among lung cancer patients [7,8]. A rapid detection of the etiological agent of infection will help in the successful management of the disease. When compared with the culture results, the rapid antigen detection test using RAPIRUN-HS® kit and the PCR did not show any significant difference ($P>0.05$). This suggests that both the RAPIRUN-HS® and the PCR test results were similar to that of the culture results. In addition, the inter-comparison between the RAPIRUN-HS® and the PCR test shows excellent correlation ($r^2=0.997$) suggesting both test methods are in close agreement with each other. Although both tests showed similar results, the time taken for the detection of *S. pneumoniae* in lung cancer patients by RAPIRUN-HS® kit was approximately 15 min, much lesser than the PCR test which takes approximately 4 h including the time taken for DNA extraction. In our study, although the sensitivity of PCR (82.6%) is higher, the RAPIRUN-HS® test showed high specificity (79.1% *vs.* 63.7%) that PCR. Similarly, the PPV of RAPIRUN-HS® is higher (78.7% *vs.* 69.7%) than the PCR. The high specificity and PPV of RAPIRUN-HS® test indicate that there may be less likely chances of misdiagnosis. Although

we did not evaluate RAPIRUN-HS® test using different serum titers, we used<100 µl of serum or plasma for the detection of *S. pneumoniae* antigen. Thus it may be possible to suggest that the RAPIRUN-HS® test can be useful in the rapid detection of *S. pneumoniae* antigen and deciding appropriate treatment for *S. pneumonia* in lung cancer patients. In addition, the high positive concordance and the strong correlation between RAPIRUN-HS® and PCR suggest that it may be useful in detecting pneumococcal infections non-lung cancer patients. However, we did not test any samples from non-lung cancer patients it is a possible assumption and needs to be validated by further studies involving diverse samples.

Infection often complicates pneumonia, infectious agents disseminate easily into the bloodstream and spreads to other body systems leading to bad prognosis. The increasing resistance of such infectious organism towards various antibiotics adds an additional burden during the treatment of pneumonia. *S. pneumoniae* one of the major causative agents of pneumonia was reported to increase its resistance towards various antibiotics such as penicillin, cephalosporins, macrolides, and fluoroquinolones [5,25]. In the United States, about 8%-15% of *S. pneumoniae* was reported to be resistant to penicillin while in Asian countries it ranges to 50% to an overwhelming 70% [26-28]. Although, *S. pneumoniae* was reported to have an increasing resistance towards penicillin none of our isolates were found to be resistant to penicillin. Similar to our findings a study from Spain reported that all the *S. pneumoniae* isolated from cancer patients were susceptible to penicillin [16]. Even though none of our isolates were resistant to penicillin, 15.2% of our isolates were found to be resistant to amoxicillin. We report that 58.7% of our isolates were found to be resistant to erythromycin which is higher than that reported (28%) elsewhere [29-31]. The overall MIC50 and MIC90 of erythromycin was 1 µg/ml and 64 µg/ml (range: ≤ 0.03 to >128 µg/ml) respectively. There were 5 isolates which showed higher resistance to erythromycin with a MIC value of >128 µg/ml. Erythromycin-resistant *S. pneumoniae* complicates the choice of antibiotic treatment since other macrolides such as clarithromycin and azithromycin, might not be an ideal choice where the prevalence of resistant pneumococci is high [27,31]. We found that resistance towards other tested antibiotics such as cefotaxime (2.2%), levofloxacin (2.2%) and cefepime (1.1%) are comparable to the earlier report [32]. Except for penicillin, our isolates showed intermediate resistance to all the other tested antibiotics which include levofloxacin (34.8%), erythromycin (22.8%), clindamycin (18.5%), cefepime (14.1%), amoxicillin (13%) and cefotaxime (5.4%). We report an overall mortality of 17.5% which is lower than that reported (30%) from France [33]. However, mortality due to infection was higher 25% when compared to the study from France [33]. Our study has few limitations which need to be acknowledged. It is a single center study and the prevalence of *S. pneumoniae* if of that particular geographical area and may not represent the entire country. Since the study population included was based on the presence of pneumonia in lung cancer patients, colonization of other microorganisms due to other infections may be overlooked. The non-availability of prior vaccination and follow-up data does not allow us to correlate our results with the pre and post clinical conditions of the studied population.

Conclusion

In conclusion, we report that *S. pneumoniae* colonization was predominantly present in pneumonia patients with lung cancer and associated with increased morbidity. The RAPIRUN-HS® is highly specific in detecting *S. pneumoniae* than the advanced molecular PCR technique. Thus RAPIRUN-HS® may be considered to a rapid and cost effective technique to be included in the diagnosis of *S. pneumoniae* in lung cancer patients. Although none of our isolates were penicillin resistance, the presence of erythromycin resistance indeed emphasizes the judicious use of antibiotic and the necessary periodic surveillance of antibiotic resistance. Further, a study involving larger patient population covering a wide range of geographical location is warranted in this patient population.

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