

Frequency of *Streptococcus pneumoniae* infection in patients with suspected meningitis in Imam Reza Hospital of Kermanshah in the west of Iran

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Received: July 2014, Accepted: December 2014

ABSTRACT

Background and Objectives: *Streptococcus pneumoniae* is the main causative agent of acute bacterial meningitis in the world. This study aimed to detect *pneumoniae* infection in cerebrospinal fluid (CSF) samples of patients with suspected meningitis.

Materials and Methods: In this study, 200 CSF samples in patients with suspected meningitis and with negative bacterial cultures were tested. Demographic data of patients and the laboratory analysis of CSF samples were also collected in a questionnaire. Pneumococcal capsular gene *spn9802* was examined by real-time PCR technique.

Results: Of 200 CSF samples from patients with the average age of 32.1±25.3 year old, 20 were positive for *S. pneumoniae* in patients with the average age of 35.05±24.6. The biochemical and cytological analysis of positive samples showed significant changes, including, 39.7 mg/DL protein, 46.2 mg/DL glucose and 1173 white blood cells per microliter of CSF on average.

Conclusion: The results of this study showed the significant pneumococcal infection in culture negative CSF samples. The majority of this infection occurred in middle-aged patients and with a higher incidence rate in the winter. It seems that the traditional methods of cultivation are not sensitive enough to detect this bacterium in CSF. Alternatively, the molecular techniques such a real-time seem to be accurate, sensitive and rapid for the detection of this agent in CSF. The cytological and biochemical findings of CSF can provide valuable clues in the diagnosis of bacterial meningitis.

Keywords: Culture-negative meningitis, real-time PCR, *Streptococcus pneumoniae*

INTRODUCTION

Streptococcus pneumoniae can cause a range of illnesses, including pneumonia, sinusitis, otitis

media, bacteremia, cellulitis, and acute bacterial meningitis (1, 2). Every year a large number of people, particularly in developing countries, lose their lives from different diseases caused by this bacterium (3,4). *S. pneumoniae* is the leading cause of acute bacterial meningitis in adults and young people (5). Meningitis caused by this bacterium has a higher rate of mortality than any other bacteria that cause this disease (16 to 37%) (6). Permanent neurological disabilities occur in 30 to 52% of surviving patients (7,8). Traditional methods used in the diagnosis of meningitis include microscopy, bacterial culture, cytology and biochemical analysis

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of CSF, and serological methods (1,2,4,9). Direct examination of smears prepared from CSF is a quick method to make a clinical decision but needs at least 10^4 to 10^5 bacteria per mL of CSF to get a positive result. This factor reduces the sensitivity of this method. As a fastidious organism, *S. pneumoniae* grows very difficult using conventional culture media and requires at least 24-48 hours time (10,11). Due to the risk of death or permanent neurological sequelae, patients with suspected bacterial meningitis usually receive antibiotics in the first medical visit prior to sampling. This can kill the bacteria in sterile fluids of the body within 2-4 hours (12).

Consequently, it might result in a negative culture of bacteria and thus reduces the sensitivity of this method to approximately 30% in medical diagnostic laboratories (13). Therefore, if the conventional bacteriology is used for diagnosis, as it occurs in many medical laboratories in Iran, the prevalence of pneumococcal meningitis is underestimated. Alternative methods based on DNA amplification using distinct target genes have been developed for the detection and rapid diagnosis of pneumococcal meningitis (11,14). Given the advantages, including the reducing of time, the elimination of post PCR processing and quantitative analysis, real-time PCR method has been updated for this purpose (15). Furthermore, the rate of possible contamination of samples is lower in real-time PCR than conventional PCR, making it more sensitive and specific technique for diagnosis (15-17). The purpose of this study was to evaluate the quantitative real time PCR assay for detection of *S. pneumoniae* from CSF specimens of patients suspected with meningitis.

MATERIALS AND METHODS

Sampling. A total of 200 culture-negative CSF samples from patients suspected with meningitis,

according to an infectious disease specialist examination were investigated in this study. Samples were collected during 2011 to 2013 from patients in the main hospital (Imam Reza) in Kermanshah city located in the west of Iran. The samples were quickly transported in an ice box under sterile conditions to the microbiology laboratory and kept at -70°C until DNA was extracted (2). CSF samples with volume less than $50\ \mu\text{L}$ and samples with positive bacterial cultures were excluded from the study.

Real-time PCR assay. The $100\ \mu\text{L}$ of CSF samples were used for DNA extraction by CinnaPure DNA kit (SinaClon, Iran) according to manufacturer's instructions. The concentration of extracting DNA (OD 260/280NM) was determined by spectrophotometry and gel electrophoresis and the specificity and characteristics of the DNA were determined using the internal control. Extracted DNA was kept at minus 20°C until used in real-time PCR assay. The following primers were used to amplify a part of *spn9802* gene (18) of *S. pneumoniae*, F: (AGTCGTCCAAGGTAAACAAGTCT) and R: (ACCAACTCGACCACCTCTTT). Real-time PCR reaction was prepared in a final volume of $20\ \mu\text{L}$, using $6\ \mu\text{L}$ of Master mix Eva Green, $0.5\ \mu\text{L}$ of each primer, $9\ \mu\text{L}$ deionized distilled water and $4\ \mu\text{L}$ of DNA template. The real-time PCR apparatus was Rotor-Gene 6000 (Corbett Research, Australia). The initial denaturing temperature of 95°C was used for 10 min followed by 40 cycles of 95°C for 30 s, and 60°C for 60 seconds.

Five serial dilutions of standard bacterial DNA contained the 10^1 to 10^5 copies per mL of bacterial genome were prepared as positive control reactions in each run of real-time PCR. At each stage, based on E (efficiency) equals to 1.02 ± 0.05 , and the amount of R_2 (Coefficient of Determination) and R (Regression) which was lower than 0.975, the test was repeated.

Table 1. The results of serial dilutions of *S.pneumoniae*(PTCC1240)

No.	Std*	CFU/mL	CT	Bacterial Count(CFU/mL)
1	S ₁	10^5	19.42	1.5e+5
2	S ₂	10^4	22.64	1.7e+4
3	S ₃	10^3	25.35	2.5e+3
4	S ₄	10^2	28.03	5.5e+2
5	S ₅	10^1	32.11	7.6e+1

Std*=Standard

Statistical analysis. All collected data were statistically analyzed using SPSS version 18 and the comparison of data was done using t-test.

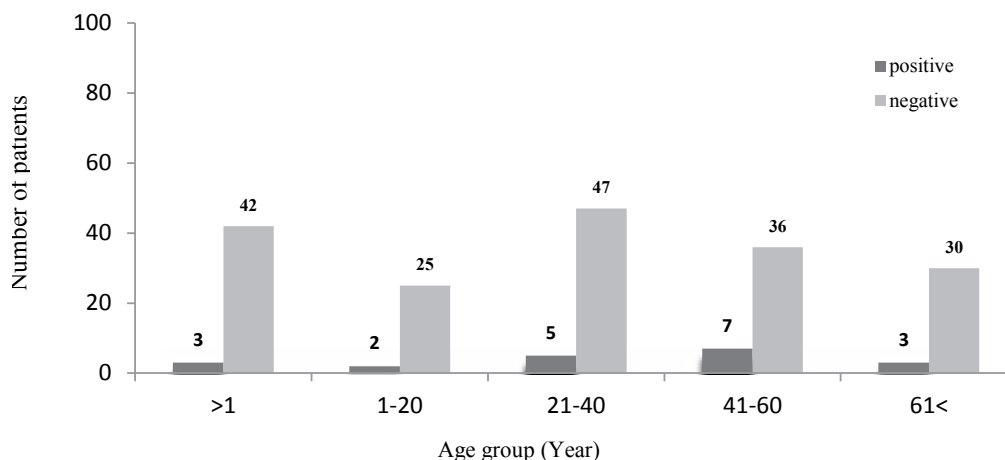


Fig. 1. The age distribution of patients with positive and negative cases.

Specificity of reaction. Genome of various bacteria including *Staphylococcus aureus* (PTCC 1189), *Enterococcus faecalis* (PTCC 1237), *Streptococcus epidermidis* (PTCC 1435), *Streptococcus agalactiae* (PTCC 1768), *Streptococcus pyogenes* (PTCC 1447); *Neisseria meningitidis* (ATCC 13090); *Escherichia coli* (PTCC 1395), *Klebsiella pneumoniae* (PTCC 1053), *Pseudomonas aeruginosa* (PTCC 1310), *Haemophilus influenzae* (PTCC 1766), *Salmonella typhi* (PTCC 1609); and gram-positive bacilli: *Listeria monocytogenes* (PTCC 1297) and human DNA were extracted and used as controls in real-time reactions.

Determination of sensitivity of reaction. To determine the sensitivity of the method, the serial dilutions of 10^1 to 10^5 copies per mL of *S. pneumoniae* (PTCC1240) genome were prepared. These serial dilutions were used for making the standard curve of real-time PCR (Table 1).

RESULTS

Of 200 cases, 99 (%49) and 101 (%51) were males and females, respectively. Their age ranged from one day to 91 year old with an average age of 35.05 ± 24.6 years. In total, 20 cases of pneumococcal infections were detected in CSF samples (Table 2 and Fig. 2). Of these, 11 and 9 were males and females, respectively, with an average age of 35.5 years old (Fig. 1). Seven cases occurred in winter that was slightly higher than other seasons. The average number of bacterial genomes in CSF samples was 2.6×10^4 copies/ml. The statistical results of the comparison of positive and negative samples are presented in Table 3.

DISCUSSION

Despite great advances in medical knowledge, bacterial meningitis remains as one of the infectious disease with high mortality rate. *S. pneumoniae* is the major cause of bacterial meningitis in all ages in many countries, although with different rates of prevalence. Our results showed 10% of culture-negative CSF was positive for *S. pneumoniae* infection. That indicates the significant rate of pneumococcal meningitis among undiagnosed CSF samples. In many cases, CSF samples are not routinely tested by molecular methods to detect this bacterium (2,9,13,14,19). Studies in different countries using PCR and real-time PCR methods, have reported the positive cases of pneumococcal infection in culture-negative CSF samples. In Brazil, of 428 CSF samples tested, 70 cases were positive (16.35%), for *S. pneumoniae* while in the United States, 5 samples out of 11 (45.45%) and in England 3 out of 7 (42.85%) samples gave positive results for this organism as causative of infection (5,19,20). Because of better primary examination and evaluation by doctors, it seems that in developed countries more cases can be detected. Conversely, in India 2 out of 43 samples (4.65%) were positive for *S. pneumoniae* in cases suspected with meningitis (14).

The conventional methods for identification of bacterial meningitis need to have at least 10^4 to 10^6 live bacteria per mL of CSF that is hard to occur because of antibiotic consumption before sampling (10,11). Our results indicated the range of bacterial genome copy was from 50 to 150000 per mL of CSF. This is mainly less than limit range for conventional culture methods and explained, at least in part, the

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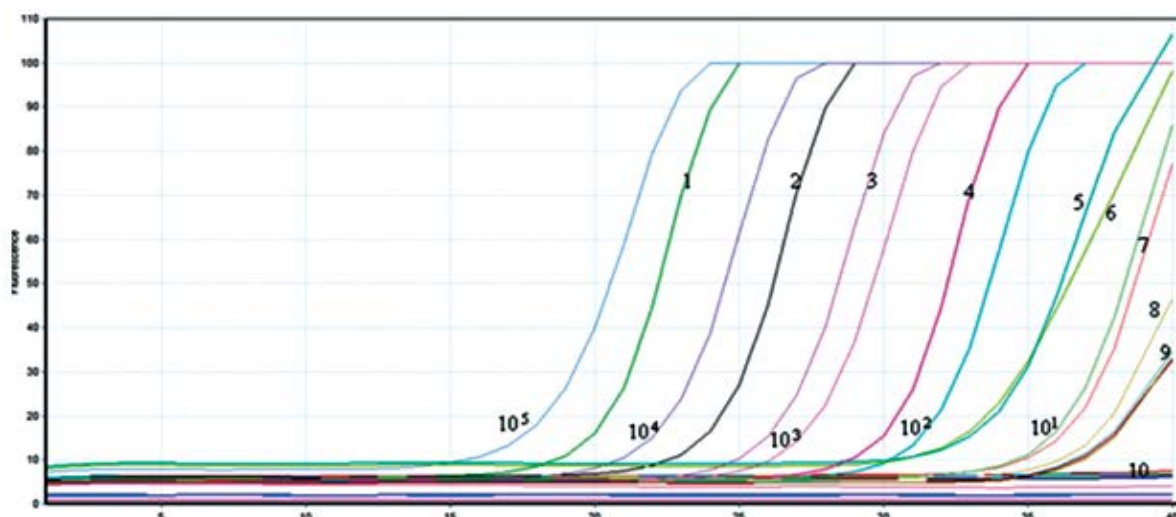


Fig. 2. Real-time PCR results. *S. pneumoniae* (PTCC1240) standard dilutions (10^5 up to 10^1), Pneumococcal positive Samples (number 1 to 6), Negative Samples (7 to 9), Negative control (10).

Table 2. Biochemical and cytological characteristics of positive samples.

Gender	Age	Protein mg/dL	Glucose mg/dL	WBC	Differential counts		CT	Bacterial count (copies/mL)
					Seg. %	Lym. %		
M	75	27	91	25	75	25	23.07	(2.7e+3)
M	73	40	41	700	70	30	20.81	(9.4e+4)
M	69	16	70	8000	90	10	19.45	(1.4e+5)
M	53	37	20	160	15	85	23.94	(1.5e+3)
M	50	65	55	8000	90	10	19.64	(1.5e+5)
M	47	32	52	200	50	50	24.27	(1.4e+3)
M	32	20	42	3200	80	20	22.83	(1.3e+4)
M	26	90	35	110	75	25	21.91	(1.5e+4)
M	19	25	36	140	20	80	26.02	(1.3e+3)
M	C*	40	20	50	70	30	30.51	(8.1e+2)
M	C*	42	66	100	90	10	20.97	(6.5e+4)
F	60	20	47	100	40	60	26.01	(1.1e+3)
F	53	65	22	900	70	30	22.64	(1.4e+4)
F	42	100	39	1350	85	15	22.59	(9.8e+3)
F	35	18	82	53	85	15	24.28	(1.4e+3)
F	24	20	55	50	82	18	24.37	(1.3e+3)
F	23	28	55	50	80	20	24.76	1.3e+3
F	19	56	23	200	70	30	25.66	(2.5e+3)
F	C*	18	43	40	60	40	33.03	(5e+1)
F	C*	40	41	40	75	25	25.48	(1.1e+3)

C*= Children less than a year old F= Female M=Male CT=Cycle threshold

Table 3. Comparison analysis of positive and negative samples.

Characteristic	Negative samples (180)	Positive samples (20)	p-value
Average age	36.23	35.05	0.24
Protein	28.27	39.95	0.001
Glucose	62.26	46.75	0.008
WBC count	203.34	1174.40	0.041

lower sensitivity of conventional methods.

In bacterial meningitis, biochemical and cytological characteristics of CSF can provide valuable clues for diagnosis. Among these characteristics, increased in the number of white blood cells (WBC), increase in protein level and the decrease of glucose level in CSF are helpful clues. However, these factors cannot unequivocally differentiate viral from bacterial cases (21,22).

In our study, a significant increase in the number of WBC and protein level and decrease in glucose level was observed, which is consistent with the medical context (26). In terms of age distribution of patients, the results of other studies indicated that the *S. pneumoniae* is the main cause of bacterial meningitis in elderly people with the age range 40 to 60 years, which is in agreement with our findings (23,24). In terms of the incidence of pneumococcal meningitis in men and women, our results showed the slightly higher rate of the positive cases among men, which is consistent with other reports (25,26). The incidence of bacterial meningitis occurs mostly in winter (26-28) and that is similar to our results. However, because of a small number of positive cases, seasonal pattern was not statistically approved.

CONCLUSION

The results of this study showed the significant pneumococcal infection rate in culture - negative CSF samples in Kermanshah. The majority of these infections occurred in middle-aged patients and with the slightly higher incidence rate in the winter. It seems that the traditional methods of cultivation are not sensitive enough to detect this bacterium in CSF. Alternatively, real-time PCR seems to be accurate, sensitive and rapid for the detection of this agent in CSF. The cytological and biochemical findings of CSF analysis, including the increase in the number of WBC and protein level and decrease in the glucose level can provide valuable clues for the diagnosis of bacterial meningitis.

ACKNOWLEDGEMENTS

This work was performed in partial fulfillment of the requirements for Master's degree of Medical Microbiology of Kamal Ahmadi, in Faculty of Medicine, Kermanshah University of Medical Sciences, Kermanshah, Iran.

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