Real-Time PCR For Direct Detection of *Streptococcus Pneumoniae* In Patients Suffering From Upper Respiratory Tract Infection in Babylon Province

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Abstract

Streptococcus pneumoniae is one of the main humans pathogens, it is important cause of community-acquired pneumoniae. It causes clinical respiratory signs in lower and upper respiratory tract. *S. pneumoniae* may also occur in the upper respiratory tract, and can be detected from nasopharyngeal secretions. In this study, we developed a Real-Time PCR specific for direct detection of *Streptococcus pneumoniae* to be applied from nasopharyngeal secretions in adult patients with respiratory tract infections. The Real-Time PCR Primers were designed from the highly conserved of 16S ribosomal RNA gene of *S. pneumoniae*. Study results, were show that Real-Time PCR was highly sensitive and specific of up to 98% in detection of *S. pneumoniae* from nasopharyngeal secretions. Where, the bacteria recorded 34 positive out of 46 specimens (73.9%). The study concluded that the used Real-Time PCR assay was provide a sensitive and reliable means for rapid detection of *S. pneumoniae* in upper respiratory tract infection and this assay may serve a suitable molecular diagnostic tool for detection of *S. pneumoniae* from nasopharyngeal secretions.

Keywords: Streptococcus pneumonia, respiratory tract infection

التشخيص المباشر لبكتريا

Streptococcus pneumoniae باستخدام تقنية RT-PCR للمرضى المصابين

بالتهابات الجهاز التنفسي العلوية والسفلية في محافظة بابل

الخلاصة

تعتبر بكتيريا *Streptococcus pneumonia* واحدة من أهم العوامل المرضية للإصابة بالالتهابات الرئوية المكتسبة والتي تظهر اعراضها السريرية من خلال التهابات وإصابة الجهاز التنفسي العلوية والسفلية والتي تم عزلها من الإفرازات التنفسية من الانف والحنجرة ، في هذه الدراسة تم استخدام تقنية RT–PCR في التشخيص المباشر للإصابات التنفسية للمرضى من عينات الافرازات التنفسية وقد تم تصميم البرايمرات الخاصة بهذه الدراسة وفق البرنامج المتبع في تصميم البرايمرات على اساس جين 16S ribosomal RNA. أثبتت الدراسة أن تقنية PCR أكثر حساسية وخصوصية في التشخيص المباشر للعينات ووصلت النسبة في هذه الدراسة إلى ٧٣,٩%من العينات ٣٤ من العدد الكلي البالغ ٤٦ عينة إفراز تنفسي من المجرى الأنفي والحنجرة للمرضى المصابين بالتهابات الجهاز التنفسي العلوية والسفلية .

Introduction

Streptococcus pneumoniae is one of the main bacterial agents of community-acquired pneumoniae [1].Also it is an important cause of both mucosal and invasive diseases including otitis media, pneumonia, arthritis, septicemia, meningitis, and sinusitis [2, 3]. The *S. pneumoniae* can also colonize the upper respiratory tract, it is generally accepted that pneumococcal infection begins with the colonization of the nasopharynx, which allows progression of *S. pneumoniae* into the lower parts of the respiratory tract, eventually leading to systemic diseases [4, 5]. Community-acquired pneumonia (CAP) caused by *Streptococcus pneumoniae* is particularly associated with high significant of morbidity and mortality rates, especially in infants and elderly, ones and is a sixth most common cause of death in the United States and posses a major economic burden of the health care system [6].

The diagnosis of *Streptococcus pneumoniae* by bacterial culture relies on the successful growth and identification of bacteria from blood cultures,and this method requires several days to yield results [7]. The limitations of culture-based detection of *S. pneumoniae* make the establishment of a definitive diagnosis to be difficult, where as the serologic assays for both antibody and antigen detection lack the specificity and sensitivity, respectivily [8]. Thus, more appropriate diagnostic tools need to be developed to improve the etiologic diagnosis of CAP caused by *S. pneumoniae*. Polymerase chain reaction (PCR) a molecular diagnostic technique was provide non-culture-based tools for a rapid identification of *S. pneumoniae* in respiratory tract specimens, pleural aspirate, and blood samples, respectivily [9]. Several studies have evaluated the use of high sensitivity of real-time PCR assays for the detection of *S. pneumoniae* DNA in blood samples of patients suspected with infection of invasive pneumococcal disease [10, 11]. However, rapid identification of bacterial diseases was providing early and appropriate pathogen oriented therapy with antibacterial drugs. The aim of the present study was to develop a method for rapid and direct detection of *S. pneumoniae* that has a high sensitivity with and specificity by use real-time PCR based syber green dye assay with primers specific for the 16S rRNA for the detection of *S. pneumoniae*.

Materials and Methods

Specimen collection: 46 nasopharyngeal secretions specimens were collected from adult patients with acute infection of upper respiratory tract from Babylon hospitals(Marjan, Al-Qasim, Al-Hashimia) at Babylon province. The samples were placed in25 ml sterile container and directly transported to the laboratory and stored in freezer until used for bacterial genomic DNA extraction.

Extraction Bacterial genomic DNA: Bacterial genomic DNA was extracted from specimens of nasopharyngeal secretions by use (PrestoTM Mini gDNA Bacteria Kit, Geneaid. U.K). A 1ml of nasopharyngeal sample was placed in 1.5ml microcentrifuge tube and rotate in a high speed cold centrifuge at 10000 rpm for 1 min, and then the supernatant was discarded. The remaining cell pellets used for genomic DNA extraction. Than, the extraction was done according to company instruction. After that, the extracted DNA was checked through Nanodrop spectrophotometer, then stored in -20C

at refrigerator until perform Real-Time PCR.

Real-Time PCR Assay

It was performed by use Syber green dye based amplification of 16S rRNA gene *Streptococcus pneumoniae*. The Primers were design in this study from *Streptococcus pneumoniae* strain KCCM 41570 16S ribosomal RNA gene, partial sequence (GenBank: KC243783.1) by using NCBI GenBank Database and the last version of Primer3 plus design online, then the primer were provided by (Bioneer company. Korea) as show in table (1).

Table (1) primer of 16 S ribosomal RNA gene

Primer	Sequence		Amplicon
16S rRNA	F	CTGTGGCTTAACCATAGTAG	91bp
	R	CTACGCATTTCACCGCTACA	

The Real-Time PCR amplification reaction was done by using (AccuPower^{TM} 2X Green star qPCR master mix kit, Bioneer. Korea) and the qPCR master mix were prepared for each sample according to company instruction as in the following table(2).

Table (2) qPCR master mix kit, Bioneer. Korea

qPCR master mix	Volume
Genomic DNA template	2.5µL
2X Green star master mix	25µL
16SrRNA Forward primer (10pmol)	1µL
16SrRNA Reverse primer (10pmol)	1µL
DEPC water	20.5µL
Total volume	50µL

These qPCR master mix reaction components that mentioned in table(2) was placed in sterile white qPCR strip tubes and then transferred into Exispin vortex centrifuge for 3minutes, then placed in MiniOpticon Real-Time PCR system and applied the following thermocycler conditions as shown in the following table(3).

Table (3) q PCR step and thermo cycler conditions

qPCR step	Temperature	Time	Repeat cycle
Initial Denaturation	95 °C	3 minute	1
Denaturation	95 °C	10 sec	45
Annealing\ Extension	55 °C	30 sec	
Detection(scan)			
Melting	60-95°C	0.5 sec	1

Results and Discussion

Real-Time PCR detection results obtained from nasopharyngeal secretions samples were positive for 34/46 (73.9%) of patients with upper respiratory tract infection. The specificity of the real-time PCR assay for *S. pneumoniae* with primers specific for the 16S rRNA gene was investigated by dissociation curve (Melt Curve). In this study the positive amplification product samples show specific amplification at melt peak mainly at (Tm: 84C°) without primer diamer or nonspecific products (Fig. 1, 2).

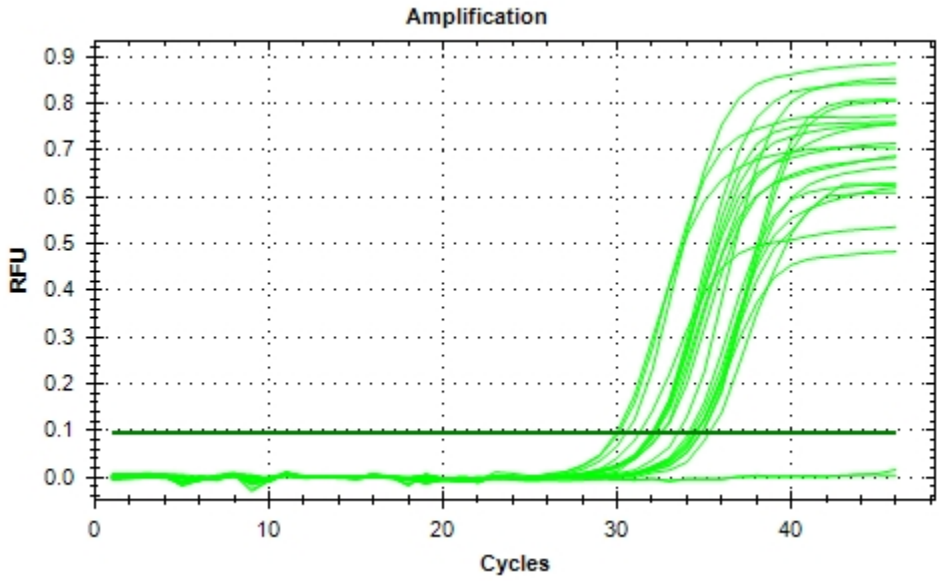

Fig. 1: Real-Time PCR amplification plots that shown the positive samples with amplification ranged from 29 to 32 cycles contained of DNA specific for *S. pneumoniae* while the negative samples which had no amplification remained under threshold line until 45 cycle.

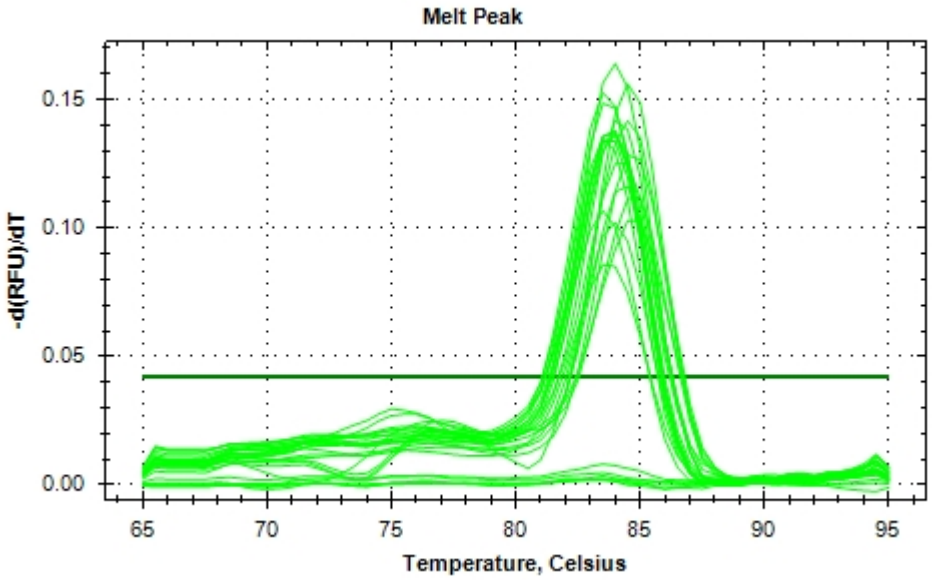

Fig. 2: Real-Time PCR Melt curve that shows the melting point for *S. pneumoniae* 16S rRNA gene ranged from 83.5°C to 84.5°C for positive samples, whereas no melting point for *S. pneumoniae* 16S rRNA gene negative samples.

The present study have established a sensitive detection method that enabled rapid detection of *Streptococcus pneumoniae* in upper respiratory tract infection, this molecular detection method was depended on the selection of specific primers for *S. pneumoniae* used in Real-Time PCR amplification, which proved to be highly sensitive (100%) and specific (up to 98%) for the detection of *S. pneumoniae* in nasopharyngeal secretions samples (Fig. 2).

The CT values, which are inversely related to the quantity of organisms was ranged between 29 and 32 at an annealing extension temperature of 55°C (Fig.1). Real-Time PCR assay for detection of *S. pneumoniae* also used by [12] who used real-time PCR that targeted the pneumolysin gene provided a sensitive and reliable method for routine rapid detection and quantification of *S. pneumoniae* present in nasopharyngeal secretions samples.

This assay may serve as a tool to study changes in the amounts of *S. pneumoniae* during lower respiratory tract infections. Other study by [13] who improved specificities of assay compared with those of currently available assays and considered the assays of choice for the detection of pneumococcal DNA. The advantages of real-time PCR over conventional assays are its rapid; elimination of the need for post processing steps which could contribute to contamination; and its wider dynamic range, which allows the detection over much larger variations in concentrations of the target. The most important advantage is the lower limit of detection, thus, the real-time PCR technology shows great sensitivity and is the only means of detection of some of the respiratory viruses [14].

The presence of *S. pneumoniae* in nasopharyngeal secretions was agreed with [15]. Who is noticed that the

bacterial adherence is regarded as the first step in colonization of this bacterium on the respiratory tract surfaces, than the pneumococci adhere to nasopharyngeal buccal epithelial cells, lung vascular endothelial , bronchoepithelial cells, and also to lung resting pneumocytes too. The present study concluded that the used of Real-Time PCR assay was provided a sensitive and reliable means for rapid detection of *S. pneumoniae* in upper respiratory tract infection and this assay may serve as a suitable molecular diagnostic tool,and as a gold standard for detection *S. pneumoniae* from nasopharyngeal secretions.

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