Full Length Research Paper

Detection and quantification of *Streptococcus pneumoniae* from Iranian patients with pneumonia and individual carriers by real time PCR

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The aim of this study was to develop a real time polymerase chain reaction (PCR) for quantitative detection of *Streptococcus pneumoniae* from clinical respiratory specimens. Initially, 184 respiratory specimens from patients with community acquired pneumonia (CAP) (n = 129) and 55 cases with hospital associated pneumonia (HAP) were bacteriologically investigated. To check the colonization status among the healthy individuals, 32 preschool and 31 adults were screened in parallel. All specimens were cultured on selective culture media to isolate *S. pneumoniae*, *Legionella* spp. and *Mycoplasma* spp. A 166 bp fragment corresponding to *cbp* A gene of *S. pneumoniae* was amplified from clinical specimens using Taqman probe real time PCR. Culture showed 14, but real time PCR showed 15 specimens as being positive for *S. pneumoniae*. The specificity and sensitivity of real time PCR was 99.14% and 100 respectively. Co-infections of *S. pneumoniae* with *Legionella pneumophila*, *Chlamydophila pneumoniae*, *Mycoplasma pneumoniae* and *Staphylococcus aureus* were observed in 5 cases (35.72%). *S. pneumoniae* was counted $<10^3$ cfu/ml from the co-infected cases. Using real time PCR, a cutoff of 10^3 cfu/ml is introduced to differentiate colonization from infection in respiratory tract. This is the first report on the prevalence CAP with *S. pneumoniae* in Iran (12.40%).

Key words: *Streptococcus pneumoniae*, community acquired pneumonia (CAP), real time polymerase chain reaction (PCR), choline binding protein A (*cbp* A).

INTRODUCTION

Pneumoniae is one of the major causes of mortalities throughout the world. *Streptococcus pneumoniae* is an important and common pathogen that is isolated from patients (30 to 40%) with community acquired pneumonia (CAP) (Morozumi et al., 2006). It can also cause bacteremia, meningitis, otitis media and sinusitis among young children and adults (Klugman et al., 2008). Rapid diagnostic methods with high specificity and sensitivity is highly demanded to manage the patients and reduce the mortality. Based on clinical finding and blood culture, the Thorax Associations in the USA and the UK have introduced guidelines for diagnosis (WHO /UNICEF, 2009; Mandell, 2007; British Thoracic Society Reports, 2009). However, rapid detection has important role in saving life of patients with severe pneumonia (WHO/UNICEF, 2009; Mandell, 2007; British Thoracic Society Reports, 2009).

The chest radiography showing alveolar consolidation and blood culture are usually used to define acute

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respiratory infections (McAvin et al., 2001). This method, however, can not show consolidation in the onset of pneumonia particularly in children and lack the sensitivity in vaccinated patients (Klugman et al., 2008).

The Thoracic Associations suggested clinical finding to be confirmed by microbiologic semi quantitative culture (Mandell et al., 2007; British Thoracic Society Reports, 2009). Culture of nasopharyngeal swab and sputum (or any samples) can be performed for this purpose. Since *S. pneumoniae* can be isolated from 5 to 40% of healthy children and 20 to 30% of adult, controversy remains over its colonization and infection since antibiotic suppression may give false negative result in culture. Moreover, contamination with normal microbiota such as alpha streptococci makes decision of administration of antibiotic, particularly to the young children, difficult too (McAvin et al., 2001). Detection of organism from the blood is considered as "gold standard" (British Thoracic Society Reports, 2009; Rudan et al., 2004), but it is labor and time consuming. Moreover, bacteremia caused by *S. pneumoniae* occurs in less than 30% of patients with CAP (Greiner et al., 2001).

Using real time polymerase chain reaction (PCR), the virulence factors of *S. pneumoniae* such as *ply* gene (pneumolysin), Spn9802, *lytA* gene (autolysin) and *wzy* gene (capsular gene) have been searched by several investigators (Carvalho et al., 2007). Yang et al. (2005) used *ply* for identification of this organism from sputum specimens with sensitivity and specificity of 90 and 80%, respectively (Yang et al., 2005).

Autolysin is a well characterized virulence factor encoded by the *lyt*A gene. The *lyt*A is conserved within species and has been used as a sensitive and specific marker for identification of *S. pneumonia*e strains (McAvin et al., 2001). Another gene, Spn9802, was used on the nasopharyngeal aspirates and reported to correlate with disease symptoms (Abdeldaim et al., 2008). These genes were also seen in some strain of S. mitis-oralis group (Whatmore et al., 2000).Although, almost clinical isolates are encapsulated and the *wzy* gene (capsular gene) for species specific detection had been used, isolates of *S. pneumoniae* lacking capsule has recently been reported (Abdeldaim et al., 2008; Bergmann and Hammerschmidt, 2006).

We developed real time PCR by amplification of *cbpA* gene to directly detect and quantify the *S. pneumoniae* from the nasopharyngeal swabs and sputum specimens collected from patients with CAP as well as healthy children and adults.

Using this method, we introduced a cutoff for differentiation of colonization from infection. Due to self-medication in Iran, it is difficult to determine the true prevalence of *S. pneumoniae* in CAP patient by conventional bacteriological techniques. Therefore, this is the first report on the role of *S. pneumoniae* in CAP among the Iranian patients and its presence with microbiota in healthy individuals.

MATERIALS AND METHODS

Study population

This is a cross-sectional study on a cohort of Iranian patients with CAP referred to teaching Hospitals (Imam Khomaini, Rasol-Akaram, Labafinezhad and Shahid Rajaee) and 3 private hospitals [Mehrad, Kasraand Shahriar Hospital) in Tehran. The inclusion criteria for the patients with suspected pneumonia were: consolidation in chest X-ray, fever (>38°C), leukocytosis (>10^4 /μL) and respiratory rate ≥ 32 breath per min. Community acquired pneumonia was defined according to the ATS and BTS guidelines(Mandell, 2007; British Thoracic Society Reports, 2009). Exclusion criteria were antibiotic consumption in less than 96 h later.

Overall, 459 patients referred to the aforementioned hospitals were monitored and 129 cases with CAP were included in this study. The nasopharyngeal swabs were used to collect samples from the randomly selected healthy individual including 32 preschool children (mean age 4.7± 0.3 years) with less than 6 years old and 16 young persons (Mean 28.4 ± 2.04) and 15 old people (mean age 58.9 ± 3.2) range 55 to 69 years old. Also, 55 patients with hospital associated pneumonia staying in intensive or critical care units (ICU or CCU) more than 48 h were subjected to *S. pneumoniae* primers and probes for monitoring *S. pneumoniae* in HAP patients.

Culture and identification

Nasopharyngeal swab of sputum from all patients with suspected pneumonia and bronco alveolar lavage (BAL) from HAP patients were cultured. The swabbed specimens were held on tubes containing 2 ml of 0.85% NaCl and vortex for 30 s. They were subsequently cultured on sheep blood agar, gentamicin blood agar and thioglycholate broth using calibrated loop (0.01 μL). The cultures were incubated in 3 to 5% CO_2 at 35 ± 1°C. The samples and clinical specimens were also cultured on buffered charcoal yeast extract (BCYE-GVPC) to screen them for *Legionella* spp. after heat treatment. In addition, samples were cultured in glucose methylene blue diphasic media for possible growth of *Mycoplasma spp.* They were also checked for *Chlamydiophila pneumoniae* using a commercial PCR diagnostic kit from Primer design, UK (http://primerdesign.co.uk/Pathogen_Detection_Kits.asp).

Before culturing, direct smears from specimens were prepared and microscopically checked after staining with Gram method. The samples were qualified as clinically significant in case the numbers of WBC were 10 cells per field of microscope at high power. Moreover, identification of *S. pneumoniae* was based on colony morphology, alpha hemolytic on sRBC blood agar, catalase negative, optochin susceptibility and bile solubility (Murray and Baron, 2007). The identification was also confirmed by capsular polyclonal serum agglutination test (Bahar Co.Tehran-Iran). Sheep blood agar plates were lawn with suspected *S. pneumoniae* morphology and an optochin disk (Hi-media, India) was placed in the center of each plate. Optochin susceptibility was defined after incubation for 18 to 24 h at 35°C in both 5% CO_2 and ambient air environments with ≥ 14 mm inhibition zone as positive (Bergmann and HammerSchmidt, 2006). After the growth, the colonies were counted in duplicate. Based on the Colony forming unit (CFUs) per milliliter, the culture count reporting for the physicians were 1,000 - 10,000 - 50,000 and 100,000.

DNA extraction

DNA was extracted using enzyme digestion and phenol chloroform method as described previously (Feizabadi et al., 2010). Five-
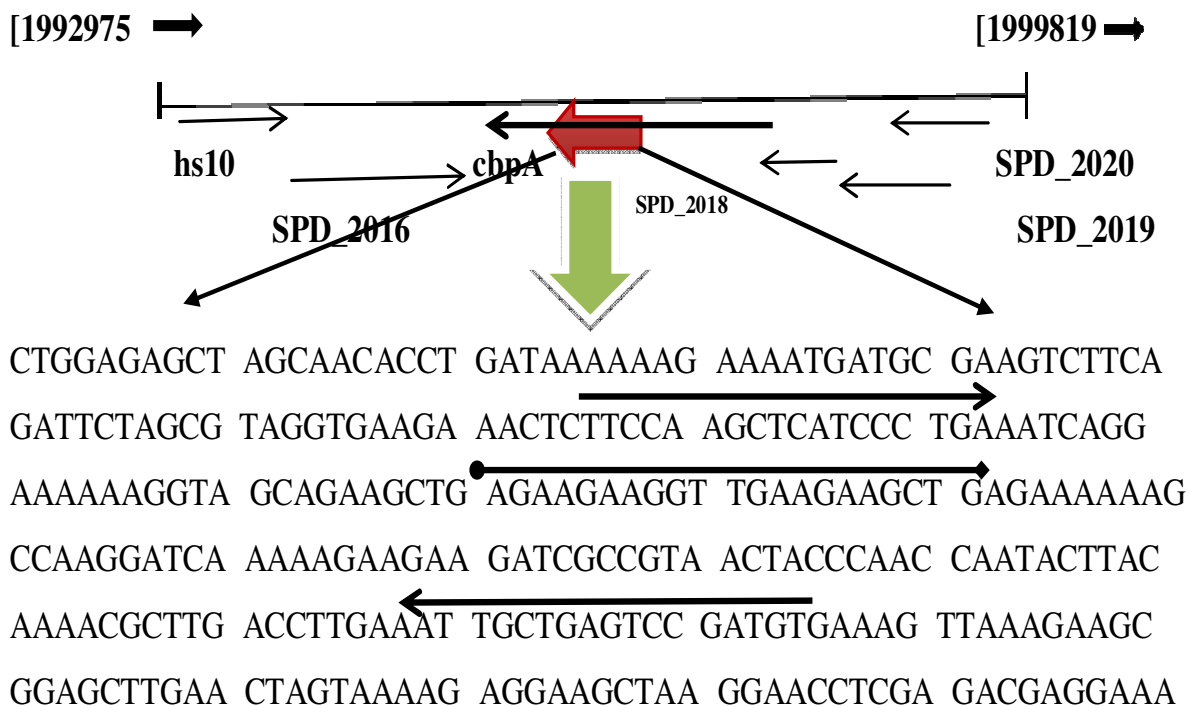
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hs10   **cbpA**   **SPD_2020**

SPD_2016   SPD_2018

                                                        SPD_2019

CTGGAGAGCT AGCAACACCT GATAAAAAAG AAAATGATGC GAAGTCTTCA

GATTCTAGCG TAGGTGAAGA AACTCTTCCA AGCTCATCCC TGAAATCAGG

AAAAAAGGTA GCAGAAGCTG AGAAGAAGGT TGAAGAAGCT GAGAAAAAAG

CCAAGGATCA AAAAGAAGAA GATCGCCGTA ACTACCCAAC CAATACTTAC

AAAACGCTTG ACCTTGAAAT TGCTGAGTCC GATGTGAAAG TTAAAGAAGC

GGAGCTTGAA CTAGTAAAAG AGGAAGCTAA GGAACCTCGA GACGAGGAAA

Figure 1. The region of cbpA gene in *S. pneumoniae* D39 (**NC_008533.1**).the amplification region was marked by red arrow.

microliter aliquots of extracted DNA solution were used as template in real time PCR assay (Feizabadi et al., 2010). The concentration and purity of extracted DNA were assessed at wavelengths 260 to 280 nm using Nanodrop (Labnet - USA).

Standards, sensitivity and specificity

S. pneumoniae (NTCC 11109) and human DNA were used as positive and negative controls, respectively. Serial dilutions of overnight culture of *S. pneumoniae* containing 10^8 to 10^2 CFU /ml were prepared in 0.85% NaCl for drawing standard curve and to determine the sensitivity of real time PCR by comparing standard tube to turbidity of 0.5 McFarland (approximate count 1.5×10^8 CFU/ml bacteria) and using spectrophotometer (Eppendorf, Hamburg, Germany) (Feizabadi et al., 2010).

When the result of turbidity of the tube and colony count was SD <1%, it was considered as standard tube of count. DNA was extracted from 200 µL of every dilution tube as earlier described (Feizabadi et al., 2010).

The concentration of extracted DNA was measured using a Nanodrop (Analytik Jena -Germany) and by using mathematical equation, it was converted to bacterial count based on standard *S. pneumoniae* genome.Bacterial DNA from *Pseudomonas aeruginosa* (ATCC 49189) *Stenotrophomonas maltophilia* (NTCC10257), *Klebsiella pneumoniae* (NTCC 5056), *Escherichia coli* (NTCC 21157), *Staphylococcus aureus* (ATCC 29213), *Streptococcus oralis* (NTCC 57342), *Streptococcus mitis* (NTCC 58274), *Legionella pneumophila* (NTCC 11192), *Mycoplasma pneumoniae* (NTCC 10119) and human DNA were used as negative controls in real time PCR. Beta actin gene (primer and probe) was used as internal control with every sample to detect PCR inhibitor as recommended (Pfaffl, 2001).

The human white blood cell DNA was used as negative control in

this study since our primers and probe do not react with human genome and positive for beta actin gene to evaluate PCR performance. The specificity of the developed real time PCR was then assessed using the aforementioned standard bacterial strains, as well as the specimens that were positive for other microorganisms. For plotting standard curve, real time PCR assays were performed in duplicate.

Primer design

The conserved sequence from cbpA (choline binding protein A) was selected as a target for real time PCR. The primers: *cbp* A-F: CTTCCAAGCTCATCCCTGAA (20bp); cbpA-R: CTTTCACAT CGGACTCAGCA (20bp); and Taqman probe (cbpA-P): FAM-GAGAAGAAGGTTGAAGAAGCTGA-BHQ1 (23bp), were designed for real time PCR assay using the strategy earlier explained (Feizabadi et al., 2010). The expected size of amplicon is 166 bp (Figure 1).

Real time PCR

Amplification and detection were performed in Line gene K (Bioer Technology Co., Ltd. - China). The reactions consisted of 0.2 µM of each primer and probe, 0.2 µM dNTP, 1.5 µM MgCl2, 1.1 U of DNA polymerase (mi Taq- Metabion, Martinsried, Germany), 2.5 µL of 10 x PCR buffer, and 5 µL of the template DNA in total volume of 25 µL with double distilled water. The cycling program was adjusted at 95°C for 10 min and then 35 cycles of 10 s each at 95°C (denaturation) followed by 50 s at 60°C with fluorescent collection (annealing and extension). Every sample was run in triplicate and mean of them was reported. The efficacy of PCR (E) was calculated using the slope of standard curve in the equation: $E = [10^{(-1/slope)}] -1$

Table 1. Distribution and clinical findings of study groups.

Patient	CAP (%)	Healthy control		HAP
		Preschool	adult	
No.	129	32	31	55
Age	12 - 71	3.5 - 6	25-69	14 - 73
Background disease	47 (36.44)	0	0	55
Chest X-Ray infiltration	108 (83.7)	ND	ND	55
Leukocytosis	113 (87.60)	ND	ND	49
Fever (≥38.0)	103 (79.84)	0	0	50
ESR(≥4.0 first hour)	99 (76.75)	0	0	35
Antibiotic consumption	16 (12.40)	0	0	55
Cough	129 (100)	0	0	54
Chest pain	126 (97.68)	0	0	55

Preschool and adults are control group; ND, not determined; ESR, erythrocyte sediment rate.

as described by Pfaffl (2001).

Statistical analysis

Comparisons of culture and real time PCR was done by using Cohen´s Kappa test. The SPSS 13.0 software program was used for Chi-square and confidence interval analysis of the data (Alonzo and Pepe, 1999).

RESULTS

Study group

All cases with CAP were diagnosed by pulmonary specialist, among them 108 cases had consolidation in chest X-ray and 19 showed infiltrations one week later. Overall, 25 of CAP patients died because of pneumonia. Antibiotic was consumed by 16 of patients < 12 hour prior to sampling. Distribution of study group and demographic feature is shown in the Table 1.

Culture semi-quantity and real time PCR

Out of 129 CAP specimens, real time PCR detected 16 positive for *S. pneumonia CbpA* gene (prevalence 12.40%). The culture was positive for 14 specimens, confirming the results of real time PCR. Semi quantification culture was in the range of 500 to 10^5 cfu per milliliter (Table 2). The result of culture for a 78 years old diabetic patient (sputum IM26) was negative at the time of admission in hospital. This patient however, died after one week with pneumonia complication. More also, *S. pneumoniae* from the specimens of 3 children in preschool control group (Nb2, Nb17 and Nb31) and 2 adults yielded growths. The organism was also detected by real time PCR from their specimens. Two of control group (Nb6 and Na18) had alpha hemolytic Streptococci

with susceptibility to optochin in their specimens. The isolated organisms from these patients bacteriologically were not identified as *S. pneumoniae* and gave no amplicons in real time PCR too.

The bacterial loads in the clinical specimens and the controls, determined by culture and real time PCR, are shown in Table 2. *L. pneumophila* (two patients), *S. aureus*, *M. pneumonia* and *C. pneumoniae* (each one case) were also isolated from 5 of patients with *S. pneumoniae* culture positive (35.71% of positives). Since the load of these bacteria was > 10,000 cfu/ml of specimens, they may be presumed as the main pathogens. These patients were under treatment with azithromycin and showed good response to therapy. Standard curve of triplicated every dilution had slope -3.29 ± 0.1 and R^2 >0.993 with SD < 0.01. Efficacy of PCR test was 101.34% ($E=10^{(-1/slope)}$ - 1) (Feizabadi et al., 2010). Results of real time PCR and quantification of every sample are shown in Table 2. Based on the results of culture, as golden standard, the specificity and sensitivity of real time PCR were 99.14 and 100% respectively. Negative predictive values of the test were 100%. Amplification of target genes from *S. pneumoniae* in serial dilutions ranged from 10^9 to 10^2 CFU /ml and showed that the technique can detect every calculated tube count with SD <1%. The results were confirmed in triplicate. There was agreement between the results of culture and real time PCR (Cohen´s Kappa value ≈1.0, SD <0.001 with p <0.001).

DISCUSSION

Annually, 1.8 million children deaths occur due to pneumonia throughout the world (Whatmore et al., 2000; CDC/MMWR, 2010; Rudan et al., 2008). In respect to importance of pneumonia, WHO designated 12th November, 2010 as World Pneumonia Day (CDC/ MMWR, 2010). In spite of progress in diagnostic

Table 2. Identification and Quantification of culture and real time PCR result.

ID	Optochin susceptibility	Bile solubility	Capsular agglutination	Culture (cfu/ml)	Real time PCR *cbp* A (cfu/ml) (mean)	Co-infection
IM3	+	+	+	$<10^5$	1.27 e + 5	
IM26	ND	ND	ND	NG	2.84 e + 2	*L. pneumophila*
IM53	+	+	+	$<10^4$	1.11 e + 4	
IM59	+	+	+	$<5\times10^4$	3.0 e + 4	
IM63	+	+	+	>1000	2.1 e + 3	*S. aureus*
M78	+	+	+	$<5\times10^4$	4.01 e + 4	
M84	+	+	-	$<10^3$	8.64 e + 2	*L. pneumophila*
B12	+	-	-	NG	0	*E. faecalis*
B27	+	+	+	$>10^5$	3.56 e + 6	
B43	+	+	+	$>10^4$	5.01 e + 4	
Ks17	+	+	+	$>10^5$	7.19 e + 7	
Ks25	+	+	+	$>10^5$	3.34 e + 6	
E59	+	+	+	<103	9.87 e + 2	*C. pneumoniae*
L31	+	+	+	$>10^5$	1.56 e + 6	
L19	ND	ND	ND	NG	6.09 e + 1	
L20	+	+	+	$<5\times10^4$	1.53 e + 5	
S46	+	+	+	<500	2.39 e + 2	*M. pneumoniae*
Nb2	+	+	+	<500	2.3 e + 2	
Nb6	+	-	-	$<10^3$	0	*(alpha Strep)*
Nb17	+	+	+	< 500	5.13 e + 2	
Nb31	+	+	+	<500	8,36 e + 2	
Na18	+*	-	-	$>10^3$	0	*(alpha Strep)*
Na23	+	+	+	<500	1.78 e + 2	
Na27	+	+	+	10^3	9.03 e + 2	

* The inhibition zone was less than 14 mm.

microbiology, difficulties in detection and differentiation of colonization and infection remain to be resolved. Presumptive identification of *S. pneumoniae* in diagnostic laboratory is based on phenotypic characteristics including colonial morphology, alpha hemolytic activity, optochin sensitivity, bile solubility and agglutination with anti-pneumococcal capsular polyclonal antibodies (Bergmann and Hammerschmidt, 2006). But recently, optochin resistance and bile insolubility have been reported for *S. pneumoniae* (Whatmore et al., 2000; Carvalho, et al., 2010). These atypical characteristic make the identification ambiguous if nasopharyngeal, sputum and other specimens are contaminated with closely related species present in the normal flora such as *S. mitis* and *S. oralis*. These normal flora 16S rRNA gene sequences show more than 90% similarities with *S. pneumonia* (Whatmore et al., 2000).

To overcome these problems, various real time PCR methods had been introduced for detecting and quantifying *S. pneumoniae* by amplification of pneumococcal virulence factors genes including autolysin (*lyt* A) (Harris et al., 2008; Loens et al., 2009; Brugger et al., 2009), pneumolysin (*ply*) (Greiner et al., 2001; Moscoso et al., 2006) and pneumococcal surface adhesion (*psa A*)

(Carvalho et al., 2007). However, there are several reports stating that *lyt* A, *ply* and *psa* A are not specific to *S. pneumoniae* and may be shared by some strains of *S.mitis* –*S.oralis* groups (Carvalho et al., 2007; Moscoso et al., 2009). This study was therefore designed to develop a novel *cbpA* based real time PCR assay to detect *S. pneumoniae* from patients and healthy control specimens. *cbpA* encodes choline binding protein which is a multifunctional pneumococcal surface protein. This protein mediates colonization of pneumococci into nasopharyngeal epithelial cells and invasion of the host cells (a major adhesin). It also interacts with components of the immune system (Carvalho et al., 2007) and play important roles in pneumococcal infections (Moscoso et al., 2006; Moscoso et al., 2009). The 3′ end region of *cbpA* was selected for designing the Oligonucleotides after aligning this gene with *cbp* A-like genes (Figure 1). The specificity of *cbp* A primers and probe was proved in challenge with standard Gram positive and Gram negative strains. In comparison with culture, this new assay showed sensitivity of 100% and specificity of 99.14%. It can detect and enumerate *S. pneumoniae* from clinical specimens in < 4 h and can help to make quick and correct decision for treatment in clinical wards.

The number of *S. pneumoniae* in sample had concordance with semi-quantitative culture (p<0.001). The specificities of real time PCR using *lyt* A, *ply* and *psa* A (up to 96%) are reported to be lower than our result (99.14%). However, all of them had similar sensitivity (100%) (McAvin et al., 2001; Greiner et al., 2001; Carvalho et al., 2007; Yang et al., 2005; Harris et al., 2008). Our real time PCR may be useful not only for monitoring of patients but also for differentiation of colonization from infection. The clinical findings and cumulative scoring clinical signs (such as CURE 65) in less than 90% of our cases confirmed pneumonia. Despite of great values of clinical findings in diagnosis of pneumonia, the infective agent should be identified to implement appropriate antibiotic therapy policy (Mandell et al., 2007; British Thoracic Society Reports, 2009). In respect to labor and time consuming of culture method for detection and identification *S. pneumonia* from clinical specimens, we suggest that real time PCR is the best substitute, since it was proved as a rapid and accurate diagnostic tool in this study.

In our study, we assessed 63 healthy persons as controls. In preschool group, only 9.38% of children gave positive results in real time PCR assay and culture. The adult group gave 6.45% positive results in the same experiments. The bacterial load calculated by real time PCR and culture in both groups was <1000 cfu/ml. In patients with bacterial load <1000 cfu /ml, other bacteria were involved as the main cause of pneumonia. Exception was patient IM63, who was co-infected with *S. aureus* (load 2.1e + 3 cfu/ml). Therefore, *S. pneumoniae* with 1000 cfu/ml load may be considered as cutoff for colonization. It is the first time to introduce a cutoff for *S. pneumoniae* in Iran. However it needs to be evaluated on larger population. *S. pneumoniae* is the most common pathogen of pneumonia in all range of age (Al-Qadi et al., 2010) and responsible for more than 40% of community acquired pneumonia (CDC /MMWR, 2010). In our study, the prevalence of *S. pneumoniae* in patients with CAP was 12.40%, which is lower than other countries in the Middle East and Asian countries (Rudan et al., 2008; Al-Ghizawi et al., 2007; Arifeen et al., 2009; Köksal et al., 2010). Agwu et al. (2006) reported incidence of *S. pneumoniae* in CAP patient referred to tuberculosis clinic (Nigeria) as 6.4% (Agwu et al., 2006). However, in a recent study from Turkey, *S. pneumoniae* was isolated from (14.7%) of CAP patients (Köksal et al., 2010). Rudan et al. (2008) also reported a prevalence of 9.6 % for CAP with *S. pneumoniae* in Kuwait, which is not significantly different from our study (P= 0.01). Self-antibiotic consumption in Iran may be a reason for lower isolation and prevalence than other countries. This self-medication may also possibly be a reason for lower prevalence of *S pneumoniae* among *pneumoniae* among healthy individuals in Iran(9.38 and 6.45%) compared with reports from other countries (up to 40% of children and 10 to 20% adults) (Rudan et al., 2004, 2008).

In this study, we entered 55 bronchoalveolar lavage (BAL) specimens from hospital associated pneumonia (HAP) that were hospitalized in Shahid Rajee Heart center admitted for heart failure disease and surgery. Culture was positive only in one patient, while real time PCR had detected *S. pneumoniae* in two patients. Hence, real time PCR of *cbp* A can be used directly for detection of *S. pneumoniae* from various clinical specimens including sputum, nasopharyngeal swab and BAL to monitor CAP, HAP and colonization in healthy individuals. We therefore recommend the evaluation of this test as multiplex real time PCR for atypical causative agent of CAP.

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REFERENCES

Abdeldaim GM, Stralin K, Olcen P, Blomberg J, Herrmann B (2008). Toward a quantitative DNA-based definition of pneumococcal pneumonia: a comparison of *Streptococcus pneumoniae* target genes, with special reference to the Spn9802 fragment. Diagn. Microbiol. Infect. Dis. 60(2): 143–50.

Agwu E, Ohihion AA, Agba MI, Okogun GRA, Okodua M, Tatfeng YM, Nwobu GO (2006). Incidence of *Streptococcus pneumoniae* Infections among patients attending Tuberculosis clinics in Ekpoma, Nigeria. Shiraz E-Medical J. 7(1).

Al-Ghizawi GJ, Al-Sulami AA, Al-Taher SS (2007). Profile of community- and hospital-acquired pneumonia cases admitted to Basra General Hospital, Iraq. East Mediterr. Health J. 13: 230-42.

Alonzo TA, Pepe MS (1999).Using a combination of reference tests to assess the accuracy of a new diagnostic test. Stat. Med. 18(22): 2987-3003.

Al-Qadi MO, Al-Alwan A, Opal SM, Cilley B, Silverblatt FJ (2010). Community acquired pneumonia. Med. Health R I. 93(7): 196-200.

Arifeen SE. Saha SK, Rahman S, Rahman KM, Rahman SM, Bari S, Naheed A, Mannan I, Seraji MH, Ahmed NU, Hassan MS, Huda N, Siddik AU, Quasem I, Islam M, Fatima K, Al-Emran H, Brooks WA, Baqui AH, Breiman RF, Sack D, Luby SP (2009). Invasive pneumococcal disease among children in rural Bangladesh: results from a population-based surveillance. Clin. Infect. Dis. 48Suppl 2: S103-113.

Bergmann S, Hammerschmidt S (2006). 'Versatility of pneumococcal surface proteins'. Microbiology, 152(Pt 2): 295-303.

British Thoracic Society Reports (2009). Guidelines for the Management of Community Acquired Pneumonia in Adults Update 2009, A Quick Reference Guide 1; 3; Available online at: http://thorax.bmj.com and at: www.brit-thoracic.org.uk/clinical-information/pneumonia/pneumonia-guidelines

Brugger SD, Hathaway LJ, Mühlemann K (2009). Detection of *Streptococcus pneumoniae* strain cocolonization in the nasopharynx. J. Clin. Microbiol. 47: 1750-6.

Carvalho DM, Pimenta FC, Jackson D, Roundtree A, Ahmad Y, Millar EV, O'Brien KL, Whitney CG, Cohen AL, Beall BW (2010). Revisiting pneumococcal carriage by use of broth enrichment and PCR techniques for enhanced detection of carriage and serotypes. J. Clin. Microbiol. 48: 1611-1618.

Carvalho Mda G, Tondella ML, McCaustland K, Weidlich L, McGee L, Mayer LW, Steigerwalt A, Whaley M, Facklam RR, Fields B, Carlone G, Ades EW, Dagan R, Sampson JS (2007). Evaluation and improvement of real-time PCR assays targeting lytA, ply, and psaA genes for detection of pneumococcal DNA. J. Clin. Microbiol. 45(8): 2460-2466.

CDC. World Pneumonia Day-November 12, 2010 (2010). MMWR,

59(43).

Feizabadi MM, Majnooni A, Nomanpour B, Fatolahzadeh B, Raji N, Delfani S, Habibi M, Asadi S, Parvin M (2010). Direct detection of *Pseudomonas aeruginosa* from patients with healthcare associated pneumonia by real time PCR. Infect. Genet. Evol. 10: 1247-1251.

Greiner O, Day PJ, Bosshard PP, Imeri F, Altwegg M, Nadal D (2001).Quantitative detection of *Streptococcus pneumoniae* in nasopharyngeal secretions by real-time PCR. J. Clin. Microbiol. 39(9): 3129-3134.

Harris KA, Turner P, Green EA, Hartley JC (2008). Duplex real-time PCR assay for detection of *Streptococcus pneumoniae* in clinical samples and determination of penicillin susceptibility. J. Clin. Microbiol. 46(8): 2751-2758.

Klugman KP, Madhi SA, Albrich WC (2008). Novel approaches to the identification of *Streptococcus pneumoniae* as the cause of community-acquired pneumonia. Clin Infect Dis. 47Suppl 3: S202-206.

Köksal I, Ozlü T, Bayraktar O, Yılmaz G, Bülbül Y, Oztuna F, Caylan R, Aydın K, Sucu N (2010). TUCAP Study Group Etiological agents of community-acquired pneumonia in adult patients in Turkey; a multi centric, cross-sectional study. Tubrek Toraks, 58(2): 119-127.

Loens K, Van Heirstraeten L, Malhotra-Kumar S, Goossens H, Ieven M (2009). Optimal sampling sites and methods for detection of pathogens possibly causing community-acquired lower respiratory tract infections. J. Clin. Microbiol. 47: 21-31.

Mandell LA, Wunderink RG, Anzueto A, Bartlett JG, Campbell GD, Dean NC, Dowell SF, File TM Jr, Musher DM, Niederman MS, Torres A, Whitney CG (2007). Infectious Diseases Society of America; American Thoracic Society Infectious Diseases Society of America/American Thoracic Society consensus guidelines on the management of community-acquired pneumonia in adults. Clin. Infect. Dis. 44(suppl2): S27-S72.

McAvin JC, Reilly PA, Roudabush RM, Barnes WJ, Salmen A, Jackson GW, Beninga KK, Astorga A, McCleskey FK, Huff WB, Niemeyer D, Lohman KL (2001). Sensitive and specific method for rapid identification of *Streptococcus pneumonia* using real-time fluorescence PCR. J. Clin. Microbiol. 39(10): 3446-3451.

Morozumi M, Nakayama E, Iwata S, Aoki Y, Hasegawa K, Kobayashi R, Chiba N, Tajima T, Ubukata K (2006). Simultaneous detection of pathogens in clinical samples from patients with community-acquired pneumonia by real-time PCR with pathogen-specific molecular beacon probes. J. Clin. Microbiol. 44(4): 1440-1446.

Moscoso M, García E, López R (2006). Biofilm formation by *Streptococcus pneumoniae*: role of choline, extracellular DNA, and capsular polysaccharide in microbial accretion. J. Bacteriol. 188(22): 7785-7795.

Moscoso M, García E, López R (2009). Pneumococcal biofilms. Int. Microbiol. Rev. 12(2): 77-85.

Murray PR, Baron Ellen Jo (2007). Manual of Clinical Microbiology.9th ed. ASM press.Washington DC. 734-748.

Pfaffl MW (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29(9): e45.

Rudan I, Tomaskovic L, Boschi-Pinto C, Campbell H (2004). WHO Child Health Epidemiology Reference Group Global estimate of the incidence of clinical pneumonia among children under five years of age. Bull World Health Organ. 82(12): 895-903.

Rudan I, Boschi-Pinto C, Biloglav Z, Mulholland K, Campbell H (2008). Epidemiology and etiology of childhood pneumonia. Bull World Health Organ. 86(5): 408-416.

Whatmore AM, Efstratiou A, Pickerill AP, Broughton K, Woodard G, Sturgeon D, George R, Dowson CG (2000). Genetic relationships between clinical isolates of *Streptococcus pneumoniae*, *Streptococcus oralis,* and *Streptococcus mitis*: characterization of "Atypical" pneumococci and organisms allied to S. mitis harboring *S. pneumoniae* virulence factor-encoding genes. Infect Immun. 68(3): 1374-1382.

World Health Organization/UNICEF. Global action plan for prevention andcontrol of pneumonia (GAPP) (2009). Wkly Epidemiol Rec. 84(43):451-2.
Available at http://whqlibdoc.who.int/hq/2009/who_f   ch_cah_nch _09.04_eng.pdf

Yang S, Lin S, Khalil A, Gaydos C, Nuemberger E, Juan G, Hardick J, Bartlett JG, Auwaerter PG, Rothman RE (2005). Quantitative PCR assay using sputum samples for rapid diagnosis of pneumococcal pneumonia in adult emergency department patients. J. Clin. Microbiol. 43(7): 3221-3226.