Title: Direct detection of *Pseudomonas aeruginosa* from respiratory samples of children with cystic fibrosis

ABSTRACT

Introduction

Pseudomonas aeruginosa is the most important cause of lung infection and major cause of morbidity and mortality among Cystic Fibrosis patients. Early detection of *P. aeruginosa* from clinical samples is a key element in patient management. Appropriate antimicrobial treatment will postpone the transition to chronic lung infection.

Methods

Sputum and deep pharyngeal swab samples (DPS) of CF patients were inoculated into conventional agar plates and identified by Matrix-assisted laser desorption/ionization-time of flight mass spectrometry. DNA was extracted with a column based QIAamp DNA mini kit from the samples. Molecular detection was done by using 23S rDNA specific primers with Taqman probe for quantitative PCR (qPCR) and *exoA* gene specific primers for in house PCR.

Results

A total number of 67 sputum and 33 DPS samples were included to the study. Median age of the patients was 6 (2-14) for DPS and 15(7-33) for sputum samples. Detection limit was 10^3 cfu/ml for qPCR which displayed 94% sensitivity and 93% specificity for sputum; 44% sensitivity and 100% specificity for DPS. In house PCR displayed 94% sensitivity and 93% specificity for sputum; 50% sensitivity and 100% specificity for DPS. Time reqired for conventional method was 48 hours , 6 hours for in- house PCR and 4 hours for qPCR.

Conclusion

Although culture is a reliable detection technique, a more rapid and sensitive way to detect *P. aeruginosa* from CF airway samples is essential. In our study which culture is accepted as a gold standard; PCR results is promising especially for sputum samples. Validation of

molecular methods is necessary before implementation in routine laboratories. Clinical implications of discrepant results between culture and PCR detection should be carefully evaluated and combining both approaches considering antibiotic treatment could be reasonable.

Keywords: *Pseudomonas aeruginosa*, Cystic Fibrosis, *exoA*, *23S rDNA*, Real-Time Polymerase Chain Reaction, Molecular Method, Culture Method

INTRODUCTION

Cystic Fibrosis (CF) is an autosomal recessive disease, caused by mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene which provides instructions for making a protein called CFTR. This protein functions as a channel across the membrane of the cell, plays an important role in the regulation of calcium activated chloride ion channels, sodium bicarbonate and aquaporin channels, sodium, and potassium transitions. Disease causing mutations in the CFTR gene alter the production, structure, and stability of the chloride channel. All these changes prevent the channel from functioning properly, that weakens the transport of chloride ions and the movement of water into and out of cells. Therefore, epithelial cells of the lungs, pancreas, and other organs produce mucus that is abnormally thick and sticky. Consequently, impaired mucus separation in the pericilliary layer reduces the excretion of viscous lung secretions and facilitates infection and colonization with bacteria. Abnormal mucus obstructs the airways and glands, leading to the symptoms of CF [1].

P. aeruginosa is the main pathogen involved in the regression of lung function in CF patients, can cause sepsis and acute infection, which can result in death, and a chronic infection that can last for years [2]. During acute infection, it releases toxins into the environment and damages the host tissue. Chronic infection is thought to be characterized by the formation of a biofilm on the surface of the airways epithelial cells containing thick mucus layer. These biofilms may be persistent despite aggressive antibiotic therapy [3-4].

According to the registered data of CF patients, *P. aeruginosa* is a predominant microorganism isolated from the respiratory tract of adult CF patients, and its prevalence increases to 20% in patients younger than 5 years old and up to 70% in patients aged 18 years. Also, it was found that pulmonary function regressed more rapidly because of chronic lung infection with *P. aeruginosa* and caused early death of CF patients [5]..

The first age in which *P. aeruginosa* was found to be positive is defined as an important predictor of prognosis. When *P. aeruginosa* is not eradicated properly children have higher risk for subsequent exacerbations [1,5]. Emerson et al[6] states that the risk of death in *P. aeruginosa* sputum culture positive patients with CF was 2.6 times higher and early intervention may help to reduce the morbidity and mortality of young patients.

Initial isolates of *P. aeruginosa* are generally not mucoid and are sensitive to antibiotics. Therefore, early eradication has been shown to be effective in delaying chronic and mucoid *P. aeruginosa* infection. The importance of the earliest possible detection in sputum supports the consensus on the importance of the eradication of *P. aeruginosa* [7].

Current diagnostic techniques are based on culture-based approaches that are time-consuming and subject to individual interpretation. A number of difficulties are encountered with culture method. Firstly, microbiological culture may not be sufficient to detect pathogens at low bacterial density or under antibiotic pressure. Secondly, *P. aeruginosa* may vary phenotypically, which may lead to misidentification (small colony variants, loss of pigment production, etc.). Third, *P. aeruginosa*, which produces biofilm, often prevents, or even inhibits its isolation by closing other gram negative and positive bacteria in culture plates under standard conditions [7].

In-house polymerase chain reaction (PCR) is one of the most popular methods for detecting pathogens. It provides the detection of pathogens by targeting the nucleic acid sequences of specific bacteria. PCR amplification products are stained with ethidium bromide and visualized in bands on electrophoresis gel [8]. Real-time PCR or quantitative PCR (qPCR) is a method that does not require agarose gel electrophoresis to detect amplified products. This method can measure the fluorescence signal generated by the added dyes or specific probes labeled at both ends, allowing the PCR products generated throughout the reaction to be continuously monitored. The fluorescence intensity is proportional to the amount of PCR amplicons [9].

The rapid detection of *P. aeruginosa* directly from clinical materials might be important to improve the life quality of the CF patient by rapid and timely intervention of colonization / infection. We analyzed the diagnostic value of two PCR methods from respiratory samples by comparing conventional culture method.

MATERIALS and METHODS

Respiratory samples (sputum and DPS) used in this study were obtained from CF patients followed in Marmara University Training and Research Hospital Chest Diseases department, and sent to the microbiology laboratory for routine examinations. The study protocol was approved by the Institutional Review Board and the Ethics Committee of Faculty of Medicine, Marmara University. An equal volume of sputazole (Thermo Scientific, USA) solution was added to the sputum samples and homogenized in a shaker incubator for 1 hour at 37 °C [10]. During the process, the samples were vortexed every 15 minutes. The resulting homogenates were transferred to 1,5 ml volume Eppendorf tubes for DNA extraction. DPS samples were processed directly.

Samples were initially screened by Gram staining and were inoculated further when > 25 leukocyte but >10 SEC per low power field. MacConkey plates, 5% sheep-blood agar plates (BioMerieux, France), and *Haemophilus* selective agar (chocolate agar including bacitracin) plates were used and plates were incubated for minimum 24 hours at 35,5 ^{0}C. If growth of colonies on petri plates are not sufficient for identification, they are allowed to incubate for up to 72 hours. After incubation, colonies formed in the agar plates were identified at the species level by Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) (BioMerieux, France). The results of studied samples by MALDI-TOF MS are obtained after approximately 30 minutes. *P. aeruginosa* 27853 ATCC strain was used for quality control.

DNA extraction was done with a column based QIAamp DNA mini kit (QIAGEN), following the manufacturer's instructions. Proteinase K treatment step in the procedure was performed at 56 ^{0}C for 3 hours. DNA of sputum samples was eluted in 200µl of elution buffer; DNA of DPS samples was eluted in 100µl of elution buffer and stored in -80^{0}C. Following DNA extraction, quality of total DNA was determined using 1% agarose gel electrophoresis.

In-house PCR was done targeting the *exoA* gene with *P. aeruginosa* specific primers (*exo*A-F-5'-*GAC AAC GCC CTC AGC ATC ACC AGC*-3'; *exo*A-R-5'-*CGC TGG CCC ATT CGC TCC AGC GCT*-3'), previously described by Xu et al [11]. The reaction mix comprised 2,5µl of 10Xbuffer, 2,5µl of $MgCl_2$, 0,5µl of each primers, 1µl of 10mM dNTP, 0.5µl of

DNA polymerase, 1µl (50-100ng) of DNA extract and the final reaction volume was completed to 25µl with free DNA water. Program consisted of 96°C for 5 min followed by 40 cycles of 96°C for 1 min, 55°C for 1 min, 72°C for 1 min, followed by a final extension at 72°C for 10 min. Visualization of in-house PCR products were performed by 1% agarose gel electrophoresis and ethidium bromide staining.

Quantitative PCR (qPCR) amplification was done using *P. aeruginosa* specific primers targeting the *23S rDNA* (Pa23F-5'-*TCC AAG TTT AAG GTG GTA GGC TG*-3' and Pa23R-5'-*ACC ACT TCG TCA TCT AAA AGA CGA C*-3') and Taqman probe (Pa23P-6-FAM-*AGG TAA ATC CGG GGT TTC AAG GCC*-TAMRA) as previously described by McCulloch et al [12]. Probe and primers specific to *GADPH* gene was used as an endogenous control (IPC-F- 5' *ACT CCT TTT GCA GAC CAC AG* 3'; IPC-R-5' *CAG TAG AGG CAG GGA TGA TG* 3'; IPC-Prob-5'-Yakima Yellow-*ATG CCA TCA CTG CCA CCC AGA*-BHQ-1-3'). The reaction mix comprised 10µl of 2XTaqMan Master mix, 0,8µl of each primers (for both of *23S rDNA* and endogenous control), 0,4µl of each prob (for both of *23S rDNA* and endogenous control) and, 1µl (50-100ng) of DNA extract and was made up to a final reaction volume of 20µl with free DNA water. The qPCR program consisted of 50 °C for 2 min, 95 °C for 15 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min with a plate read after every cycle. The amplification efficiency and sensitivity range of PCR assays improved were demonstrated by amplifying aliquots of 10-fold serial dilutions (10^8 to 10^2) from DNA of *P. aeruginosa* 27853 ATCC strain.

RESULTS

A total number of 67 sputum and 33 DPS samples from children with CF were included to the study. Median age was 6 (2-14) for DPS and 15(7-33) for sputum samples. In 33 of 35 sputum samples, culture and molecular methods were positive when two samples were positive for both PCR methods when the culture was negative (Table 1).

Table1. The comparison of PCR and culture results for sputum samples

Sputum	***exoA* positive**	***exoA* negative**	***23S rDNA* positive**	***23S rDNA* negative**
Culture positive	33	2	33	2
Culture negative	2	30	2	30
Total	35	32	35	32

In house PCR was negative in 9 and qPCR was negative in 10 of 15 culture positive DPS samples (Table 2).

Table2. The comparison of PCR and culture results for DPS samples.

DPS	***exoA* positive**	***exoA* negative**	***23S rDNA* positive**	***23S rDNA* negative**
Culture positive	9	9	8	10
Culture negative	0	15	0	15
Total	9	24	8	25

When the culture was taken as the gold standard, sensitivity and specificity for both PCR methods were 93-94% in sputum samples. However, we were not able to amplify targeted genes by both PCR methods in half of culture positive DPS samples and detection sensitivities were found very low (Table 3).

Table 3. Evaluation of results in-house and qPCR when the culture is taken as the gold standard.

		Sensitivity	Specificity	Positive predictive value	Negative predictive value
qPCR	**Sputum**	94%	93%	94%	93%
	DPS	44%	100%	100%	60%
In-house PCR	**Sputum**	94%	93%	94%	93%
	DPS	50%	100%	100%	62%

The time required for in-house PCR analysis, including DNA isolation, amplification, and gel electrophoresis for imaging, took approximately 6 hours whereas results were obtained in 3-4 hours by qPCR. Isolation and identification of *P. aeruginosa* in culture plates took between 24 and 48 hours, in cases requiring further incubation it took up to 72 hours.

DISCUSSION

While PCR amplification has been routinely used for diagnosis of viral infections directly from clinical samples it is not very common for bacterial infections. Early detection of *P. aeruginosa* will lead to a rapid and timely intervention to colonization/infection, leading to an increase of quality of life of the CF patients. Deschaght et al. performed a nationwide study a decade ago and stated that both routine culture techniques and DNA amplification techniques perform equally well in detection of *P. aeruginosa* in respiratory samples of CF patients [13]. For a total of 852 samples, 26 samples were negative by culture but positive by qPCR, and 10 samples were positive by culture but remained negative by qPCR. Authors concluded that qPCR may have a predictive value for impending *P. aeruginosa* infection for only a limited number of patients.

The sampling method strongly influence the sensitivity of infection diagnosis. Any definition of first life time/early/new as well as chronic *P. aeruginosa* infection should clearly describe sampling frequency [14]. Using multiple target genes improves the sensitivity of the method by eliminating false negative and false positive results. Sensitivity is not an issue when the bacterial load is high, but in low concentrations as may be found in recently colonized patients it is very important since early detection is crucial. In our study, we were not able to amplify targeted genes by both of PCR methods in half of culture positive DPS samples. This might be interpreted as insufficient bacterial presence and consequently insufficient DNA for PCR. According to a similar study of Xu et al. targeting *exoA* target gene, discrepancy in the detection of PCR and culture of *P. aeruginosa* is based on a number of factors including phenotypic misidentification and auxotrophic mutations [11]. In the study of McCulloch et al. the sensitivity value was 100% and the specificity was 52% [12]. Based on the results of a similar study by Logan et al., these data suggest that PCR may have a potential to detect *P. aeruginosa* earlier, but that the presence of the organism can be reliably reported using a combined approach of culture and molecular techniques [15]. Boutin et al. focused on the superiority of molecular techniques over culture technique which is considered the gold standard and observed a good sensitivity of the qPCR (>95% in sputum and >90% in the throat swabs) but specificity was low (83.9% in sputum and 78.6% in the throat swabs). Their results show that the discriminative power of qPCR was interpreted as high in sputum samples and low in throat swabs [16]. Low discriminative power of qPCR in throat swabs was mostly due to the low bacterial burden observed in this material However, Heltshe et al.

stated that pharyngeal culture technique results might not be generalizable to lower airway *P. aeruginosa* since pharyngeal cultures have poor sensitivity and positive predictive value for the presence of *P. aeruginosa* [17].

We detected the presence of P aeruginosa in 6 hours by in-house PCR and 4 hours by qPCR. A patient who gives a sample in the morning will be able to get the results during the day and the doctor will have the opportunity to start treatment immediately. This is a potential advantage for clinicians in order to reduce the exacerbations with early diagnosis and treatment applications of infection by *P. aeruginosa* in CF patients. It is not known whether it is reasonable and acceptable to recommend a therapeutic intervention based on a PCR result without antibiotic susceptibility data. However, early detection by PCR has the potential to increase the levels of awareness of the upcoming infection. It is also important to monitor such patients who exhibit PCR positive / culture negative findings from their sputum, to determine if there are transient infections of cases that do not result in established colonization leading to chronic infection. In addition, it is important to monitor patients with such a PCR positive / culture negative result in terms of optimal antibiotic management and infection.

Conclusion: Progressive loss of pulmonary functions related persistent colonization of *P. aeruginosa* is a big challenge for clinicians and rapid and sensitive detection of *P. aeruginosa* is the major point of patient management. If equipment is available in routine laboratories , PCR which is applied directly from clinical materials could be used for the fastest results . Early PCR detection might facilitate patient screening however it is reasonable to advise therapy based on susceptibility data.

REFERENCES

1. Talwalkar JS, Murray TS. The approach to Pseudomonas aeruginosa in cystic fibrosis.Clin Chest Med2016;37:69-81
2. Bhagirath AY, Li Y, Somayajula D, Dadashi M, Badr S, Duan K. Cystic fibrosis lung environment and Pseudomonas aeruginosa infection. BMC Pulm Med 2016;16:174
3. Jansen G, Mahrt N, Tueffers L, et al. Association between clinical antibiotic resistance and susceptibility of Pseudomonas in the cystic fibrosis lung. Evol Med Public Health 2016;2016:182-194
4. Jørgensen KM, Wassermann T, Johansen HK, et al. Diversity of metabolic profiles of cystic fibrosis Pseudomonas aeruginosa during the early stages of lung infection. Microbiol 2015;161:1447-1462
5. Ehsan Z, Clancy JP. Management of Pseudomonas aeruginosa infection in cystic fibrosis patients using inhaled antibiotics with a focus on nebulized liposomal amikacin. Future Microbiol 2015;10:1901-1912
6. Emerson J, Rosenfeld M, McNamara S, Ramsey B, Gibson RL. Pseudomonas aeruginosa and other predictors of mortality and morbidity in young children with cystic fibrosis. Pediatr Pulmonol 2002;34:91-100
7. Hery-Arnaud G, Nowak E, Caillon J, et al. Evaluation of quantitative PCR for early diagnosis of Pseudomonas aeruginosa infection in cystic fibrosis: a prospective cohort study. Clin Microbiol Infect 2017;23:203-207
8. Alahi MEE, Mukhopadhyay SC. Detection Methodologies for Pathogen and Toxins: A Review. Sensors 2017;17(8):1885
9. Deschaght P, De Baere T, Van Simaey L, et al. Comparison of the sensitivity of culture, PCR and quantitative real-time PCR for the detection of Pseudomonas aeruginosa in sputum of cystic fibrosis patients. BMC Microbiol 2009;9:244
10. Xu J, Moore JE, Murphy PG, Millar BC, Elborn JS. Early detection of Pseudomonas aeruginosa–comparison of conventional versus molecular (PCR) detection directly from adult patients with cystic fibrosis (CF). Ann Clin Microbiol Antimicrob 2004;3:21
11. McCulloch E, Lucas C, Ramage G, Williams C. Improved early diagnosis of Pseudomonas aeruginosa by real-time PCR to prevent chronic colonisation in a paediatric cystic fibrosis population. J Cyst Fibros 2011;10:21-24

12. Deschaght P, Schelstraete P, Lopes dos Santos Santiago G, et al. Comparison of culture and qPCR for the detection of Pseudomonas aeruginosa in not chronically infected cystic fibrosis patients. BMC Microbiol 2010;10:245
13. Taccetti, G., Denton, M., Hayes, K., ECFS-CTN Microbiology Group, Drevinek, P., Sermet-Gaudelus. A critical review of definitions used to describe Pseudomonas aeruginosa microbiological status in patients with cystic fibrosis for application in clinical trials. J Cyst Fibros 2020;19:52-67
14. Logan C, Habington A, Lennon G, Cronin F, O'Sullivan N. Evaluation of the efficacy of real-time polymerase chain reaction for the routine early detection of Pseudomonas aeruginosa in cystic fibrosis sputum and throat swab specimens. Diagn Microbiol Infect Dis 2010;68:358-365
15. Boutin S, Weitnauer M, Hassel S, et al. One-time quantitative PCR detection of Pseudomonas aeruginosa to discriminate intermittent from chronic infection in cystic fibrosis. J Cyst Fibros 2018;17:348-355
16. Heltshe SL, Khan U, Beckett V, et al. Longitudinal development of initial, chronic and mucoid Pseudomonas aeruginosa infection in young children with cystic fibrosis. J Cyst Fibros 2018;17:341–347
17. Saiman L, Burns JL, Larone D, Chen Y, Garber E, Whittier S. Evaluation of MicroScan Autoscan for identification of Pseudomonas aeruginosa isolates from cystic fibrosis patients. J Clin Microbiol 2003;41:492-4

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