Original Article

Comparison of Gomori's Methenamine Silver Method with PCR Technique on Oral Swab, Bronchoalveolar Lavage and lung Homogenate Specimens in Detection of *Pneumocystis*

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Abstract

Background: *Pneumocystis carinii* is one of opportunistic organisms especially in immuno-compromised patients. For years, diagnosis of *Pneumocystis* pneumonia (PCP) has relied on microscopic visualization of organism in specimens obtained from the lung either by bronchoalveolar lavage (BAL) or by induction of sputum, but failed to provide efficient diagnosis. The aim of this study was to compare of PCR result and Gomori's methenamine silver (GMS) of invasive and noninvasive specimens in infected rat.

Methods: Thirteen stimulated rat with methylprednisolone acetate used as test group and 4 non-stimulated rats were used as control group. Oral swabs (OS), BAL and lung homogenate (LH) specimens were collected from weeks 0, 2, 4, 5, 6 and 7. All samples were tested with (GMS) staining method and PCR technique.

Results: The results showed the sensitivity of PCR method higher than GMS (69.2% versus 0%) in oral swab samples (P< 0.001). The analysis of the results also proved that PCR test using noninvasive oral swab had comparable results with the GMS staining method on invasive lung biopsy specimens (69.2% versus 61% and 84.6 for BAL and Lung Biopsy).

Conclusion: Higher sensitivity of PCR using noninvasive sample can made it a useful method in rapid diagnosis of PCP.

Keywords: Pneumocystis carinii, *PCR*, *Pneumonia*, *GMS*

Introduction

*P*neumocystis pneumonia (PCP) is a world wild distributed disease. More than 75% of the children less than 4 years old have been in face with changing in serum titration. Pneumonia still is the most important opportunistic disease especially in patients with immuno-deficiency syndrome by activating of endogenous organisms (60-80%) (1-3).

Despite the use of various staining methods (4-5), one of the major problems of the clinical laboratory is diagnosis of PCP. Low efficiency of staining methods and culture in isolation and identification of the organisms caused failure diagnosis of PCP. Therefore researchers have tried to introduce alternative methods (6).

Recently, PCR method has been applied for clinical specimens with different protocols. Reported sensitivity and specificity was 100% in some cases (7). Sensitivity of PCR-hybridization has reported one organism per μl in BAL specimens of HIV positive patients. Single PCR and Nested-PCR had lower sensitivity in oral swab. Applied target genes were 23s rRNA of mitochondria, timidylate syntetase, dehydrofolate reductase and major surface glycoprotein (MSG) (8-11).

The aim of this study was to apply PCR for oral swab, bronchoalveolar lavage and lung homogenate specimens and compare the efficiency of

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PCR results with GMS staining method in detection of *Pneumocystis*.

Materials and Methods

Animal Model, Keeping Conditions and Preparation: Seventeen female rats (Sprague-Dawley) of weight of 150-200 g and two months age were selected randomly and prepared from Razi Vaccine And Serum Research Institute. They all were kept for a week to ensure lack of any bacterial contamination. They feed with UV treated food and sterile water containing 1 mg/L tetracycline. Thirteen rats out of 17 were selected as test group and the rest were used as control group after one week incubation. Methylprednisolone acetate (Depo-Medrol, Pharmacia & Upjohn Co., Belgium) was injected subcutaneously (40 mg/kg) once a week up to the sampling time to stimulate pneumocystis pneumonia in the lungs of test group.

Specimens: Oral swab, BAL and lung homogenate specimens were collected on weeks zero (before injection), 2, 4, 5, 6 and 7 from test and control groups. These samples were divided into two equal volumes for staining and PCR test.

Staining: Slide smears were prepared with 10 ul of specimens and stained with CDC procedure (12). Positive results were determined by observation of brown cyst (4-8 µM) with specific clue of GMS staining that seems to green blue with two dark spot inside (13).

Extraction Method and PCR test: Template DNA Extraction Kit KIA (Spin Kiagen Co.,) Iran was used to extract DNA from specimens. PAZ102-E and PAZ102-H primers (14-15) targeted 346 base pair of *mtlsu* rRNA genome was successfully evaluated with BLAST program. The PCR mixture was amplified using of Eppendorf Mastercycler gradient thermocycler (Table 1) and visualized with transilluminator after staining.

Statistical Method: Fisher Exact test was applied to compare the results of PCR with staining method.

Results

Sensitivity and Specificity: Three hundreds and forty six base pair of the products was observed after PCR. The lowest detection limit of the test was detected 100 fg DNA (Fig. 1). Examination with usual organisms encountered in the respiratory tract were detected no positive band.

Staining Results: Staining of the samples with GMS revealed lung homogenate specimens had higher sensitivity than BAL and oral swab samples. Positive cases were 11 (84.5%), 8 (61.7%) and 0 (0%) in lung biopsy, BAL and oral swab respectively. Control group was negative for all three kind tested specimens (Table 2).

PCR Results: PCR test showed BAL and lung homogenate were positive in 12 out of 13 specimens (92.3%) while oral swab were positive in 9 samples (69.2%). Control group was negative in oral swab and positive in 1 of the BAL and 3 of lung homogenate (Table 2). Results of Statistical Fisher Exact test revealed PCR methods as more sensitive than staining procedure ($P< 0.001$). Comparison of BAL and lung homogenate specimens showed that these two methods had no significant differences.

Table 1: Applied amplification program

Denaturation	2 min at 94° C Cycle No. 1
Denaturation	30 sec at 94° C Cycle No. 2 to 40
Annealing	30 sec at 55° C Cycle No. 2 to 40
Extension	30 sec at 72° C Cycle No. 2 to 40
Final Extension	5 min

Table 2: Comparison of PCR and staining results in three applied specimens and both test and control groups

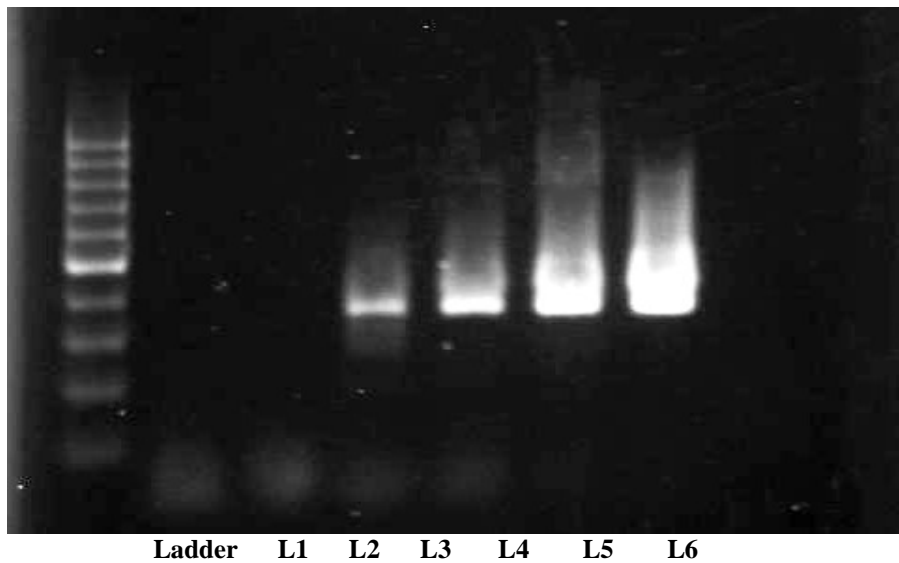|	Oral Swab				BAL				Lung Biopsy			
	Test		Control		Test		Control		Test		Control	
	PCR	Staining	PCR	Staining	PCR	Staining	PCR	Staining	PCR	Staining	PCR	Staining
No. P/c	9	0	0	0	12	8	1	0	12	11	3	0
No. N/c	4	13	4	4	1	5	3	4	1	2	1	4
Total	13	13	4	4	13	13	4	4	13	13	4	4

Fig. 1: Sensitivity of the applied PCR protocols. Lane No. 6:100 pg, Lane No.5:10pg, Lane No. 4: 1 pg, Lane No. 3: 100 fg, Lane No.2:10 fg, Lane No.1:1 fg, Ld: Ladder.

Ladder   L1   L2   L3   L4   L5   L6

Discussion

One of the major problems in clinical laboratories is rapid diagnosis of the *Pneumocystis* in clinical specimens. Diagnosis of the PCP is usually made in acute stage of the disease, when the patients are faced with high mortality rate. Therefore early diagnosis is quite important in those patients with critical conditions. Unfortunately, it is frequently reported that staining methods have low sensitivity even in invasive samples (16). This point has been stressed out especially in negative HIV patients (2, 7).

As it is reported, GMS has higher sensitivity than Giemsa in all specimens (13). In the present study, PCR test was compared with GMS staining method. Analysis of the results revealed PCR had higher sensitivity rate than GMS in oral swab tested samples. On the basis of the Table 2, PCR test on the noninvasive sample (OS) has comparable result with invasive (BAL and LH) sample provided from GMS method. Therefore, it can be used as a suitable alternative method for GMS staining method on invasive specimens. Comparison the PCR test with GMS method on all specimens individually revealed all positive stained samples had positive PCR test as well. We did not observe any cases with negative PCR results having positive GMS results even with low count of cyst. This point confirms the lower sensitivity of the GMS than PCR.

Analysis of the results also revealed PCR was positive in control group (3 in lung homogenate and 1 in BAL). In this experiment we considered negative control for each PCR test. We did not observe any contamination in all PCR experiments. Therefore, positive PCR results must have other reasons. The only possible cause that can be considered is respiratory transmitted organism in control group from those rats of the test group since they are all kept in near to gather. This point has been emphasized by Davi *et al*. (2). They reported transmitted exogenous organisms to be an important cause of infection as well as endogenous organisms. This point confirms the high sensitivity of the PCR for diagnosis of those non-stimulated rats.

These results show that the PCR using oral swab as noninvasive specimen can be a suitable method in those negative HIV patients (pregnant women, COPD, transplant, cancer patients) that have low organism count and even in those patients who unable to provide invasive samples (children, old people, patients with sever respiratory infections and patients with hematological and autoimmune diseases (15-16). PCP is currently diagnosed in late stage of the disease that patients are faced with high mortality rate (17-18). Sampling from the lung in this situation has high risk. Patients are not able usually to provide the BAL specimens.

In conclusion, application of PCR in PCP patients has quite important role to reduce mortality rate in high risk patients including AIDS and especially in negative HIV patients who have low count of the organisms. Therefore it is recommended to design other study to evaluate the efficiency of the PCR for human infections.

Acknowledgements

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