Diagnosis of Pneumocystosis pneumonia with two staining methods using various specimens collected from animal model

M Hajia, A Mahmoodzadeh, M Rezaiemanesh, H Morovati

Citation

M Hajia, A Mahmoodzadeh, M Rezaiemanesh, H Morovati. *Diagnosis of Pneumocystosis pneumonia with two staining methods using various specimens collected from animal model*. The Internet Journal of Microbiology. 2007 Volume 5 Number 2.

Abstract

Correct diagnosis of pneumocystosis pneumonia depends on suitable sampling procedure and staining method. The aim of this study was comparison two staining methods “Giemsa and GMS” in three collected samples from studied animal model “oral swab, BAL and lung homogenate”.

Twenty two female rats of two month-ages (Sprague-Dawley) with 150-200 grams of weight were used. Methyl prednizolone acetate was used to stimulate pneumocystosis. Oral swab (OS), broncho-alveolar lavage (BAL) and lung homogenate (LH) were collected during studied weeks from control and test groups. All samples were stained with Giemsa and GMS (Gomori's Methenamine Silver). These specimens were classified in four stages based on 35 microscopic fields (100X) for counting of cyst numbers, tested by two staining methods for common specificity such as number of cysts, required time for observations, cost, and possibility of improper diagnosis.

All controls were negative in all specimens by both staining methods. Oral swabs were negative by both staining methods. The Lavage specimens were negative from weeks zero to five by Giemsa method but were positive from week two to the end by GMS method. Lung homogenate specimens were negative in weeks 0 and 2 by Giemsa but negative in week 0 by GMS. These samples were positive in later weeks in two staining methods.

Based on the analyzed results Giemsa and GMS had no enough sensitivity in oral swab specimens. The best sensitivity was obtained by GMS use of LH specimens.

INTRODUCTION

Direct observation of Pneumocystis cyst or trophozoite is required for diagnosis pneumocystosis in clinical samples by staining method. Gram-Wright, Papanicolaou, Toluidine blue and GMS has been used for identification of cysts or trophozoites in prepared specimens on tissue sections. Those staining procedures using immunoflurescence monoclonal antibody have increased sensitivity and specificity ([1]). Giemsa and GMS are the most applied methods to diagnosis of PCP ([2,3]), while Giemsa has been the most rapid and cheapest method. The organisms are diagnosed in all steps of their life cycle. Those cysts with and without organisms not only can differentiate by Giemsa but can also differentiate these cysts from yeast cells ([4,5,6]) although isolation of the organisms on cell line can be an alternative procedures ([7]).

GMS is a silver staining method that is usually used for the observation of fungi in tissue specimens. This method is the specific procedure for the cyst wall and is used as a gold standard method for diagnosis of the pneumocystosis ([8,9,10,11]).

Proper diagnosis depends on applied staining method, kind of specimens and properly sampling from infected area. Specimens are provided by either non-invasive methods (such as sputum or stimulation procedure) or even invasive methods like broncho-alveolar lavage, lung homogenate and lung biopsy. Invasive methods have higher sensitivity ([12]). Those samples collected from HIV positive patients have also higher sensitivity because of high load of the organisms in these samples ([13]).

With looking through advantages and disadvantages these two methods, we want to evaluate the efficiency of the Giemsa and GMS in diagnosis of the PCP in animal model in different samples and applied conditions.

MATERIALS AND METHODS

Animal model: Twenty two female rats of two month-ages (Sprague-Dawley) with 150-200 grams of weight was provided from Razi Vaccine & Serum Research Institute. 17 rats for tests that divided in six groups and 5 rats for control that one specified for each group except zero group ([14]).

Stimulation of pneumocystosis proliferation in rat lung: methyl prednizolone acetate (40mg/ml) made by Pharmacia & Upjohn provided from Belgium was used sub-cutaneously to stimulate pneumocystosis in all rat lung of test group to increase the Pneumocystis carinii ([14]).

Specimens: Oral swab (OS), broncho-alveolar lavage (BAL) and lung homogenate (LH) were collected at weeks zero (before injection), 2, 3, 4, 5, 6, and 7 from test and control groups. All samples were divided in 1 ml micro tubes and kept at -70oC.

PROCEDURE FOR COLLECTING SPECIMENS

Oral swabs: Oral specimens were obtained by sterile applicator from mouth area; above and beneath the tongue, under Bio-safety cabinet. Cotton head of the swabs were suspended inside 5 ml of sterile PBS in sterile capped tube. The tubes were vortexed for 1 min and swabs were taken out. Specimens were centrifuged for 15 min at 3000 RPM and top 4 ml of supernatant were taken out and the rest were transferred in sterile1.5 ml tubes and kept at -70°till to be used ([14]).

Broncho-alveolar lavage: Separated lung of the rats was put in sterile Petri-dish. 2.5 ml of Dithiothreitol and 2.5 ml PBS were injected through trachea inside the lung. Lavage fluid were then collected and incubated for 15 min at 37 oC. 2.5 ml of 159 mMol (0.85%) Ammonium Chloride solution were added to the fluids and kept at laboratory temperature for 30 min. Specimens was centrifuged for 15 min at 3000 RPM. Top of supernatant was taken out and the rest 1 ml was transferred in sterile 1.5 ml tubes and kept at -70°till to be used ([15]).

Lung homogenate: Lung homogenate specimens were prepared from whole left lobe of the lung using sterile homogenizer containing 5 ml sterile PBS. Obtained fluids were transferred from sterile 3 layer of gauze filter. 2.5 ml of Dithiothreitol were added and incubated for 15 min at 37 oC. 2.5 ml of ammonium chloride (0.85%, pH 6.00) were added to the fluids to lyses of the host and blood cells and kept at laboratory temperature for 30 min. These samples were centrifuged for 15 min at 3000 RPM. Top of supernatant were taken out and the rest 1 ml were transferred in sterile 1.5 ml tubes and kept at -70°till to be used ([16]).

STAINING METHODS

Giemsa: Slide smears were prepared with 10 ul of specimens and stained with Giemsa after dry up the smears. Positive results are determined by observation of Pneumocystis cysts looking either of these clues:

- Eight circular bodies (4-8 µM)
- Purple banana shape (sporozoite) along each other
- Observation of trophozoite (2-4 µM) with blue nucleolus and pink cytoplasm that are usually in mass shape near each other or separated

Gomori's Methenamine Silver: Slide smears were prepared with 10 ul of specimens and stained with CDC procedure ([15]). 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

Counting method for Pneumocystis cysts: Slide smears were prepared with 10 ul of OS, BAL and LH. It was spread in 1 cm2 and fixed with methanol and stained with GMS or Giemsa. 35 microscopic fields in the center of the smears were counted and average of the cyst number was registered for each sampling and staining method.

Scoring system for Giemsa and GMS staining method: Average counting of 35 microscopic fields with 100 magnification were classified less than 10 cysts=1+, 11-100 cysts 2+, 101-1000 cysts 3+ and for more than 1000 cysts 4+

Considered criteria for Staining methods: Two methods were compared with each other with following criteria: No. of observed cysts (low , high), counterstaining (low, high), capability of the staining the cysts, required time for observations, cost (more than 5000Rls=high, 1000-5000 Rls = moderate, less than 1000 Rls= low), required time for smear preparation (less than 5 min= low, more than 5 min high), requirement to skilled microscopist, possibility of wrong diagnosis (lack of wrong diagnosis = negative, some wrong diagnosis cases= 1+, high wrong diagnosis cases= 2+), and requirement to specific facilities.

RESULTS

OBSERVED CYSTS BY GIEMSA STAINING METHOD

Oral swab specimens: No cysts were observed in all test and control groups during studied weeks after examining 35 microscopic fields (Figure No1).

Figure 1

Picture 1: Percentage of Positive cases in studied weeks and tested specimens with Giemsa staining method

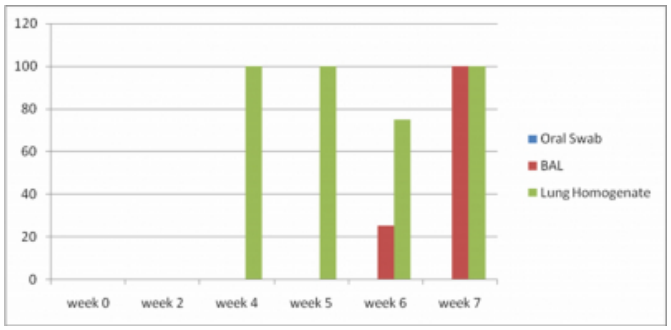


Figure 2

Picture 2: Percentage of positive cases in studied weeks and tested specimens with GMS staining method

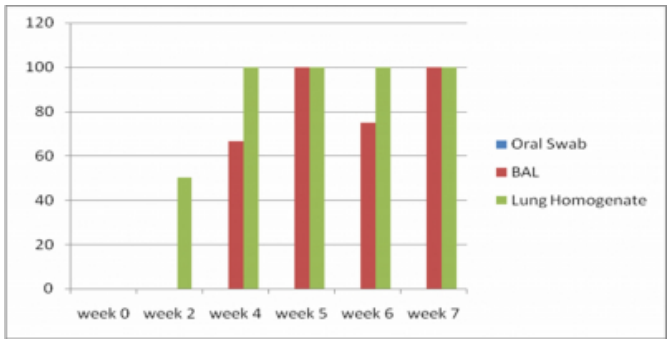


Figure 3

Figure 3: Average of estimated cysts of tested samples in control and test animal groups during weeks by Giemsa method

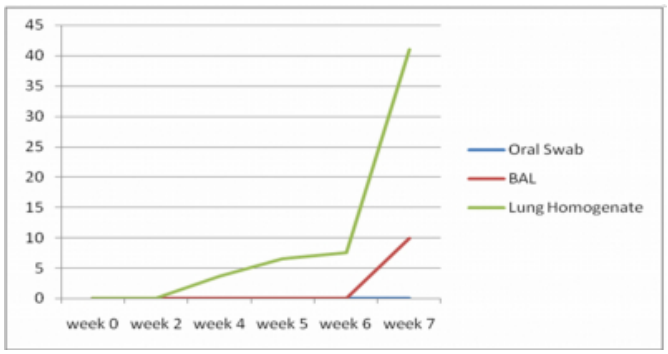
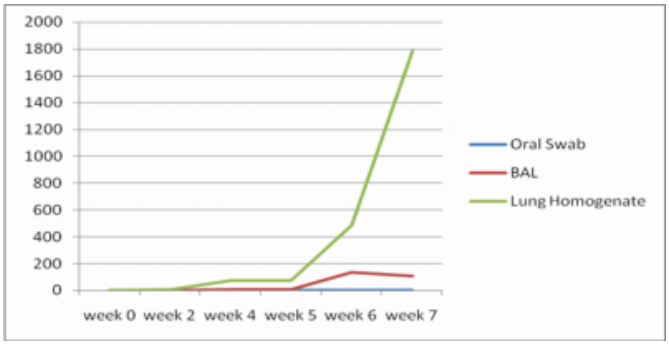


Figure 4

Figure 4: Average of estimated cysts of tested samples in control and test animal groups during weeks by GMS method



BAL specimens: Examined BAL specimens of control group in all weeks and test group collected in weeks zero, 2, 4, and 5 were negative while 25% of week six and 100% in week seven were positive (Figure No. 1).

Lung homogenate specimens: Specimens of test group collected in weeks zero and 2 and all control group specimens were remained negative. Those specimens collected from weeks 4, 5, and 7 were 100% positive while samples in week six were 75% positive (Figure No. 1).

OBSERVED CYSTS BY GMS STAINING METHOD

Oral swab specimens: No cysts were observed in all test and control groups during studied weeks after examining 35 microscopic fields (Figure No 2)

BAL specimens: Examined BAL specimens of control group in all weeks and test group collected in weeks zero and 2 were negative while 66.6% of week four,100% of week five, 75% of week six and 100% in week seven were positive (Figure No.2).

Lung homogenate specimens: Specimens of test group collected in weeks zero and all control group specimens were remained negative. That specimens collected from week 2 was 50% and weeks 4, 5, 6 and 7 were 100% positive (Figure No.2).

Estimation of Pneumocystis cysts in all three collected samples by both methods: Oral swab samples have no cysts were observed in all groups during studied weeks. In BAL Samples 10 cysts in average were observed only in week seven by Giemsa while less than 10 cysts in weeks 4 and 5 and more than 100 cysts in weeks 6 and 7 by GMS method. Observed cysts were different in lung homogenate

specimens in studied weeks by Giemsa and GMS method. In Giemsa method, no cysts were observed in weeks zero and 2, less than 10 cysts were estimated in weeks 4, 5 and 6. Estimated cysts in week seven were 10 to 100. In GMS method no cysts were observed just in week zero. Its number were less than 10 in week 2 and 10 to 100 in weeks 4, 5 and 100 to 1000 in week six and were more than 1000 in week seven (Figures No 3 and 4).

Comparison of specific criteria and scoring of two applied staining methods: considered criteria were compared with each other and methods were scored, the results are shown in (Table No.1).

Figure 5

Table 1: Comparison of the specificity of two applied staining method

	No. of observed cyst in same positive sample	Counterstaining	Staining of the cyst	Staining of the trophozoite	Require time for staining	Require time for observation	Cost of materials	Cost of time	Requirement to microscopist	Possibility of wrong diagnosis	Requirement to special equipment
GMS	High	Low	Positive	Negative	20 min	10-20 min	High	Low	No needs	Low	No needs
Giemsa	Low	High	Negative	Positive	35 min	1-5 min	Low	High	Needs	High	No Needs

Figure 6

Table 2: Percentage of Scoring in diagnosis method of Giemsa and GMS

	-	+	++	+++	+++
Giemsa	71.8	23.1	5.1	0	0
GMS	51.3	12.8	15.4	15.4	5.1

SCORING RESULTS OF STAINING METHODS

71.8% of tested specimens were negative and the rest 28.2% had just 1+ and 2+ grade by Giemsa. Negative rate of GMS was less than Giemsa. 51.3% of GMS had negative results while 15.4% and 5.1% had 3+ and 4+ grades (Table No. 2).

DISCUSSION

Three specimens (oral swab, lung homogenate and BAL) have been compared with each other in this study. Analyzed results revealed none of oral swab samples were positive even in the presence of the infection. These samples had the highest positive rate in lung homogenate and then in BAL specimens just in the early stage of the infection, while both had the same sensitivity in the last weeks.

It is frequently reported that microscopic examination of stained smears on specimens is standard method for diagnosis of PCP. Staining method is usually based on diagnosis of the cysts, because trophozoites are normally mistaken with counter-stain materials ([13, 17]). Giemsa method is the easiest, cheapest and the most rapid procedure, enable to differentiate those empty cysts from those containing organisms or even with yeast cells ([5,6]). Despite these advantages, diagnosis of the PCP is rather difficult and requires skilled microscopist because differentiation of the organisms from smears materials is not easy ([17]). Sensitivity of this method is also expected to be lower than GMS because of incapability of those empty cysts.

On the other hand, GMS method is usually used to observed fungi in tissue specimens. This method is specific for cyst wall and is applied as a gold standard method for diagnosis of pneumocystosis. Having long steps of staining and requirement of more than one hour time to perform are of its limitations. This method also can not diagnose trophozoite. There is possibility infection can not be identified in those mild infections or those cases with high ratio of trophozoite to cyst ([6,7]). Misdiagnosing is other limitation ([1]). Additionally, effect of anti-pneumocystosis drugs can not be determined because those empty cysts are not differentiated from the cysts containing trophozoites.

It is frequently observed HIV positive patients had pneumocystis cysts in their lung after treatment. Sporozoites of these cysts that have been destroyed by the drugs are responsible for these situations ([18]). We can differentiate pneumocystis cysts from other organisms because of two dark spots attached to each other inside the cysts, if GMS staining method to be performed in best conditions. We could decrease require necessary time for staining steps up to 30 min in this study.

It is reported Giemsa method has low sensitivity among studied staining procedures ([8, 17,19]). Obtaining results in this study is in agreement with the other reports. Analyzed Results revealed GMS has higher sensitivity than Giemsa. Gurpreet has reported that routine staining method has enough sensitivity in those specimens containing high organisms such as biopsy, BAL specimens and taken specimens of those HIV positive patients ([11, 20]). Obviously, this routine method has high false positive results in HIV negative patients even in BAL specimens ([15, 21,22]).

The standard staining method is microscopic observation of Pneumocystis in specimens such as BAL, Stimulated sputum, or biopsy ([23,24,25]). Oral swab sampling is non-

invasive method that has been applied in animal model ([26,27]) with no positive results. Diagnosis of PCP is usually made by stimulated sputum sampling in HIV positive patients that is rarely positive in HIV negative patients with immunodeficiency problem. Correct diagnosis requires proper sampling such as BAL or lung biopsy. However, stimulated sputum sampling is more applicable in HIV positive patients because of higher load of organisms in this patients group ([27,28,29]). The sensitivity will be increase in those invasive samples. The quality of the result will be better in those invasive specimens that sampling is near to the colonized area ([11, 30]). BAL sample is an invasive method that is occasionally facing with danger, although is a selective sample ([31]). Its sensitivity by staining methods is about 55-78 percent ([11, 32]). False negative is even high in BAL sample in HIV negative patients ([21,22]). Besides false negative rate is high in non-invasive sampling methods and those patients that are in early stage of the disease ([14]). Open lung biopsy is the most invasive method that require for operating room. Use of this technique enables us to have the best specimens, although patients are facing with side effects ([12]).

CONCLUSION

Staining is necessary for diagnosis of pneumocystis if no other alternative method is available. Therefore, it is underlining that Giemsa procedure to be considered as the last alternative method. However, it is recommended to use other new diagnostic method such as PCR to decrease the risk of danger invasive sampling.

References

1. Ribes JA, Limper AH, Espy MJ, Smith TF. PCR Detection of Pneumocystis carinii in bronchoalveolar lavage specimens: analysis of sensitivity and specificity. J. Clin. Microbiol. 1997;354:830-835.
2. Hogan TF, Riley RS, Thomas JG. Rapid diagnosis of acute eosinophilic pneumonia AEP in a patient with respiratory failure using bronchoalveolar lavage BAL with calcofluor white CW staining. J. Clin. Lab. Anal. 1997;11:202-207
3. Kaiser K, Rabodonirina M, Picot S. Real time quantitative PCR and RT-PCR for analysis of Pneumocystis carinii hominis J. Microbiol. Meth. 2001;45:113-118
4. Bigby, TD, Mrggolskee D, Curtis JL, Michael PF, Sheppard J, Hadley WK, and Hopewell PC. The usefulness of induced sputum in the diagnosis of Pneumocystis carinii pneumonia in patients with the acquired immunodeficiency syndrome. Am. Rev. Respir. Dis. 1986;133:515-518.
5. Domingo J, Waksal HW. Wright's stain in rapid diagnosis of Pneumocystis carinii. Am. J. Clin. Pathol. 1984;81:511-514.
6. Ng VL, Gartner I, Weymouth LA, Goodman CD, Hopewell PC, Hadley WK. The use of mucolysed induced sputum for the identification of pulmonary pathogens associated with human immunodeficiency virus infection. Arch. Pathol. Lab. Med. 1989;113:488-493.
7. Mahmoodzadeh Abbas, Hajia Massoud, Morovati Hassan. Use of Razi Bovine Kidney Cell-line for Proliferation of Pneumocystis carinii. Iranian J. Pathology. 2009. In Press.
8. Procop GW, Haddad S, Quinn J, Wilson M L, Henshaw NG, Reller LB. Detection of Pneumocystis jiroveci in Respiratory Specimens by Four Staining Methods. J. Clin. Microbiol. 2004;427:3333-3335.
9. Raab, SS, Cheville JC, Bottles K, and Cohen MB. Utility of Gomori methenamine silver stains in bronchoalveolar lavage specimens. Mo. Pathol. 1994;7:599-604.
10. Naimey, GL, Wuerker RB. Comparison of histologic stains in the diagnosis of Pneumocystis carinii. Acta Cytol. 1995;39:1124-1127.
11. Flori P, Bellete B, Durand F, Raberin H, Cazorla C, Hafid J. Comparison between real-time PCR, conventional PCR and different staining techniques for diagnosing Pneumocystis jiroveci pneumonia from bronchoalveolar lavage specimens J. Med. Microbiol. 2004;53:603-607.
12. Cushion MT. "Pneumocystis carinii. In: Topley and Wilson's Microbiology and Microbial Infections. Eeds. Collier L, Balows A, Sussman M. 9th Ed. Arnold and Oxford Press. New York. 1998, pp 645-683.
13. Saric M. Detection and Characterization of Ornithine Decarboxylase Activity in Rat Pneumocystis carinii: Implications for Anti-Pneumocystis Therapy.desertation for requirements for the degree of Doctor of Philosophy at New York University. 1992.
14. Mahmoodzadeh A, Hajia M, Rezaiemanesh MR, Morovati H. Score system of Pneumocystis pneumonia based on clinical symptoms and laboratory findings in rat model. Iranian J. Pathology. 2007;4:165-170.
15. Mahmoodzadeh A, Hajia M, Rezaiemanesh MR. Comparison of Gomori's Methenamine Silver Method with PCR Technique on Oral Swab, Bronchoalveolar Lavage and lung Homogenate Specimens in Detection of Pneumocystis. Iranian J. Parasitology. 2008;3(2):21-25.
16. Morovati H. Mahmoodzadeh A. Paykari. Cultivation of Pneumocystis carinii on Cell Lines. Kowsar Medical Journal. 2006;10(4);263-270.
17. Cregan P, Yamamoto A, Lum A, VanDerHeide T, MacDonald M, Pulliam L. Comparison of four methods for rapid detection of Pneumocystis carinii in respiratory specimens. J. Clin. Microbiol. 1990;28:2432-2436
18. Van Voorhis, WC. Therapy and prophylaxis of systemic protozoan infections. Drugs. 1990; 402:176-202.
19. Turner D, Schwarz Y, Yust I. Induced sputum for diagnosing Pneumocystis carinii pneumonia in HIV patients: new data, new issues. Eur. Respir. J. 2003;21:204-208.
20. Sandhu GS, Kline BC, Espy M.J, Stockman L, Smith TF, Limper AH. Laboratory Diagnosis of Pneumocystis carinii Infections by PCR Directed to Genes Encoding for Mitochondrial 5S and 28S Ribosomal RNA. Diagn. Microbiol. Infect. Dis. 1999;33:157-162
21. Flori P, Bellete B, Durand F, Raberin H, Cazorla C, Hafid J, Lucht F, Sung RTM. Comparison between real-time PCR, conventional PCR and different staining techniques for diagnosing Pneumocystis jiroveci pneumonia from bronchoalveolar lavage specimens. Journal of Medical Microbiology. 2004;53:603-607.
22. Palladino S, Kay I, Fonte R, Flexman J. Use of real-time PCR and the LightCycler system for the rapid detection of Pneumocystis carinii in respiratory specimens Diagnostic Microbiology and Infectious Disease. 2001;39 233-236.
23. Lundgren B, Wakefield AE. PCR for detecting

diagnosing Pneumocystis carinii in clinical or environmental samples. FEMS Immunol. Med. Microbiol. 1998;22:97-101.
24. Cartwright CP, Nelson NA, and Gill VJ. Development and evaluation of a rapid and simple procedure for detection of Pneumocystis carinii by PCR. J. Clin. Microbiol. 1994;32:1634-1638.
25. Leibovitz E, Pollack H, Moore T, Papellas J, Gallo L, Krasinski K. comparison of PCR and Standard Cytological Staining for Detection of Pneumocystis carinii from Respiratory Specimens from Patients with or at High Risk for Infection by Human Immunodeficiency Virus. J. Clin. Microbiol., 1995; 3311 3004-3007.
26. Icenhour CR, Rebholz SL, Collins MS, Cushion MT. Widespread occurrence of Pneumocystis carinii in commercial rat colonies detected using targeted PCR and oral swabs. J. Clin. Microbiol. 2001;39:3437-41.
27. Huang L., Morris A., Limper A.H., Beck J.M. An Official ATS Workshop Summary: Recent Advances and Future Directions in Pneumocystis Pneumonia PCP. The Proceedings of the American Thoracic Society. 2006;3:655-664
28. Kovacs J A, Ng VL, Masur H, Leoung G, Hadley WK, Evans G. Diagnosis of Pneumocystis carinii pneumonia: improved detection of in induced sputum with use of monoclonal antibodies. N. Engl. J. Med. 1988; 318:583-593.
29. Davi T. Fisk 1, Steven Meshnick, and Powel H. Kazanjian1. Pneumocystis carinii Pneumonia in Patients in the Developing World Who Have Acquired Immunodeficiency Syndrome. Clin. Infect. Dis. 2003; 36:70-8
30. Pinlaor S, Mootsikapun P, Pinlaor P, Phunmanee A, Pipitgool V, Sithithaworn P. PCR diagnosis of Pneumocystis carinii on sputum and bronchoalveolar lavage samples in immuno-compromised patients. Parasitol. Res. 94: 213-218
31. Broaddus C, Dake CM, Stulberg M S, Blumenfeld W, Hadley WK, Golden JA. Bronchoalveolar lavage and transbronchial biopsy for the diagnosis of pulmonary infections in the acquired immunodeficiency syndrome. Ann. Intern. Med. 1985;102:747-752.
32. Zaman, MK, Wooten OJ, Suprahamanya B, Ankobiah W, Finch PJP, Kamholtz S L. Rapid noninvasive diagnosis of Pneumocystis carinii from induced liquefied sputum. Ann. Intern. Med. 1988;109:7-10.

Author Information

M. Hajia, Ph.D.
Associate Professor, Medical Microbiology, Research Center of Health Reference Laboratory of Iran

A. Mahmoodzadeh, Ph.D.
Associate Professor, Medical Parasitology, Dept and Research Center of Molecular Biology, Baqyatallah Medical Sciences Univ

MR Rezaiemanesh, M.Sc.
Medical Parasitology, Microbiology Dept., Baqyatallah Medical Sciences Univ

H. Morovati, M.Sc.
Medical Parasitology, Razi Vaccine & Serum Research Institute