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PCR in diagnosis of pneumocystosis of rats

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Abstract: Polymerase chain reaction (PCR) is a powerful technique to detect scanty amount of DNA from living organisms. The present study intended to develope specific primers for PCR diagnosis of pneumocystosis and to evaluate diagnostic efficacy by preparation of template DNAs from invasive BAL fluid and also to screen serum or blood as a non-invasive specimen. Albino rats of Wistar or Fischer strains were experimentally infected by *Pneumocystis carinii*. Extracted DNAs or cell lysates of their blood, bronchoalveolar lavage fluid, and lung homogenate were used as the template DNA. Primers were synthetic oligonucleotides among 16s rDNA sequences. All of the primer combinations gave PCR products, but the primer pair of #24 and #27 gave best quality product of 666 bp. The sensitivity of PCR with lysates of BAL fluid was 57.7% but it increased to 84.6% with extracted DNAs. None of BAL lysate or DNA was positive among 13 microscopically negatives. The serum DNAs were positive only in 2 cases out of 20 morphologically positive rats. DNAs of human, rat, other parasites, yeast, and microorganisms were negative. The findings suggest that the present primers are specific but simple lysate of BAL fluid is not sensitive. PCR may be used as a routine diagnostic method of pneumocystosis if simple and rapid preparation of non-invasive clinical specimens are available.

Key words: rat, *Pneumocystis carinii*, BAL, serum, PCR

INTRODUCTION

Rapid and non-invasive diagnosis is important for adequate management of the patients of *Pneumocystis carinii* pneumonia. Since most of the patients are suffered from respiratory difficulties, bronchoscopy is only limitedly available though bronchoaveolar lavage (BAL) fluid is the choice of diagnostic specimen. Therefore, it is necessary to develope the diagnosis with other non-invasive specimens than BAL fluid.

At present, diagnosis of pneumocystosis depends upon clinical manifestations and detection of the organism in bronchoalveolar lavage fluid, sputum, or the lung tissue. Immunological procedures are of no value in diagnosis (Beard and Navin, 1996).

DNA amplification by PCR (polymerase chain reaction) is widely used to verify scanty amount of specific DNA molecules. Although PCR was recorded as a promising candidate for diagnosis of *P. carinii* pneumonia (Cartwright *et al.*, 1994), this is not adopted as a regular diagnostic measure of pneumocystosis. To apply PCR in diagnosis of pneumocystosis, the preparation procedure of template DNA should be simple and rapid. DNA extraction from *P. carinii* takes times and is laborious. Also PCR itself should be consistent. The reaction is influenced greatly

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by the amount of *P. carinii* DNA and contaminating host DNA.

The present study screened PCR with serum and lysates or extracted DNAs of BAL fluid in rat pneumocystosis. The primers were oligonucleotides of 16s rDNA (Edman *et al.*, 1988). Also cross reaction with DNAs of host or other microorganisms was screened.

MATERIALS AND METHODS

Experimental pneumocystosis of rats

Albino rats of Wistar or Fischer strains were weekly injected 4 mg of methyl prednisolone (Upjohn Korea Co. Ltd., Korea) over 6 weeks. They were bred with regular commercial diet and 0.1% tetracycline water in the conventional animal quarter of the Seoul National University College of Medicine.

Preparation of *P. carinii* for PCR

Blood of the rats was collected in tubes under ether anesthesia, and their lungs were removed. The serum was separated and DNA was extracted by phenol and chloroform treatment. The left lung was examined by impression smear for screening of *P. carinii* and the right lung was transbronchially washed out with saline. The cellular component of the bronchial washing was collected also for this study. The lungs were homogenized and *P. carinii* organisms were purified as previously described (Hong, 1991). The *P. carinii* isolates or cells from bronchial washings were lysed by overnight incubation at 55°C in lysing solution (proteinase K 0.25 mg/ml, 0.125 M EDTA, 50 mM Tris-HCl, 1% sarkosyl). The lysates were stored at -70°C and used for PCR. Also a part of the lysates were saved for DNA extraction by successive phenol and chloroform treatment and ethanol precipitation. The extracted DNAs were also stored at -70°C until use.

Template DNA of other organisms

To evaluate the cross reactivity with other organisms, the genomic DNAs from *Leishmania major, Entamoeba dispar, Anisakis simplex, Ascaris lumbricoides*, sparganum (plerocercoids of *Spirometra erinacei*), *Gastroconger myriaster*, rat, and human were screened. Microbial organisms, *Streptococcus pneumoniae, Staphylococcus aureus, Neisseria sicca, Escherichia coli, Candida albicans*, and *Saccharomyces cerevisiae* were also screened.

Preparation of primers

The primers were prepared by synthesis of oligonucleotides in laboratory of the Korea Biotech Inc. The primers used in this study were chosen among rDNA sequences as summarized in Table 1 (Edman *et al.*, 1988).

PCR

The reaction volume was 30 or 20 μl. The reaction mixtures were 1 × reaction buffer (50 mM KCl; 10 mM Tris-HCl, pH 8.3; 1.5 mM $MgCl_2$, 0.01% gelatin), dNTP mix 200 μM, primers 20 pM, template DNA 10 ng, and *Taq* polymerase 2.5 u (Boehringer Mannheim, Germany).

Three cycles were repeated 30 to 35 times in

Table 1. Primers of 16s rDNA for PCR

ID of primers	No. of oligonucleotides	Location (Edman *et al.*, 1988)	Sequences from 5' to 3'
Sense primers			
#21	23 mers	from 681 to 695 plus *Eco*RI site	GGG AAT TCA CTG ATC CTT CCC TC
#22	18 mers	from 684 to 701	GAT CCT TCC CTC CTG GAT
#23	20 mers	from 721 to 740	GGT GTG CCG GAT AGC CAG GG
#24	22 mers	from 721 to 742	GGT GTG CCG GAT AGC CAG GGC A
#25	26 mers	from 1349 to 1374	CCA GAT TAG CTT TTG CTG ATC GCG GG
Antisense primers			
#26	13 mers	from 1348 to 1360 plus *Eco*RI site	GGG AAT TCA AGC TAA TCT GGC
#27	22 mers	from 1365 to 1386	CCC TCT AAG AAG CCC GCG ATC A
#28	21 mers	from 1679 to 1699	AAT AAC CCA TCA CCA GTC CGA
#29	18 mers	from 1706 to 1723	TCC TGA AT AGG CTT ATC

heat blocks of the Thermal Cycler or Thermal Cycler 9600 (Perkin Elmer Cetus). Using the Thermal Cycler, standard 3 cycles were every 2 minutes at 94°C, 60°C, and 72°C. In the Thermal Cycler 9600, the first step was continuous keeping for 5 minutes at 94°C, and cyclic temperature change was repeated 30 seconds at 94°C for denaturation, 40 seconds at 62°C for annealing, and 40 seconds at 72°C for extension. After completion of the cycling, the tubes were kept at 72°C for one minute. Other several conditions of annealing temperature and annealing time were tried by combination of primers.

The PCR products were loaded on 0.7% agarose gels and stained with 0.1% ethidium bromide. The DNAs were observed on a UV transilluminator.

RESULTS

PCR products with several combinations of primer pairs

Five sense primers and 4 antisense primers were paired in all possible combinations. Most of the tested primer pairs gave PCR products except for the pairs of #21 with #25 or #28. However, the pair of #24 and #27 primers produced the best amplification (data not shown). This primer pair was the standard in the present study.

PCR amplification by the amount of template DNA

The present PCR condition with the primer pair #24 and #27 produced the 666 bp amplification until template *P. carinii* DNA was diluted up to10 fg (Fig. 1). When a cloned DNA of rRNA was tested, the DNA amount of 0.1 fg could produce the PCR (data not shown). Although the host rat DNA of different amount was added into the *P. carinii* DNA, the PCR still gave the right product even 100 ng of host DNA was mixed (data not shown).

PCR amplification with DNAs of other organisms

The primers #23 or #24 paired with #27 were tried but showed no products of PCR amplification with template DNAs of the rat, human, fish, yeast, helminth, or protozoa (Fig. 2). All of common microbes in the oral cavity or respiratory mucosa also showed no product with these primers.

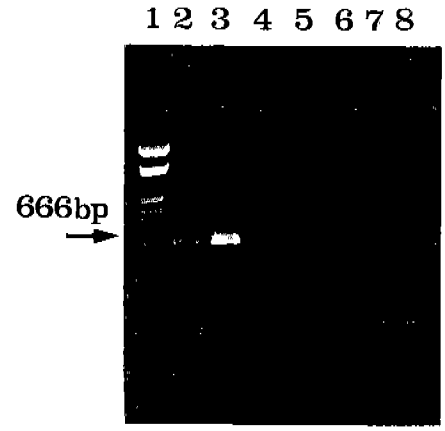

Fig. 1. PCR products of 666 bp by various amounts of template DNA in a reaction tube. The template DNA was extracted DNA of purified *P. carinii* (W13-20). Reaction volume was 50 μl and the reaction parameters were 94°C 30 sec., 61°C 30 sec., 72°C 40 sec. in a thermal cycler 9600. 1, size marker; 2, 10 ng of DNA; 3, 1 ng of DNA; 4, 0.1 ng of DNA; 5, 10 fg of DNA; 6, 1 fg of DNA; 7, 0.1 fg of DNA; 8, 0.01 fg of DNA. The lane 3 showed best reaction, and the lane 5 was marginally positive.

PCR amplification with serum, BAL fluid lysates or DNA, and extracted DNAs of *P. carinii*

All of purified *P. carinii* DNAs from rat lung homogenates were amplified. Also most of the extracted DNAs of BAL fluid of infected rats were amplified but the proteinase K lysates were amplified only in 15 out of 26 positive specimens. Only 2 sera showed positive reactions out of 20 specimens which were *P. carinii* positive in their lungs (Fig. 3). None was positive out of 6 blood DNAs which were heavily infected in their lungs.

Comparison of PCR with morphological identification

Table 2 summarized the results of PCR and of microscopic observation. All of 13 microscopic negative BAL fluids showed no products by PCR. Sensitivity of PCR with extracted BAL fluid DNA was 84.6% and that with BAL lysates was 57.7%. Specificity was

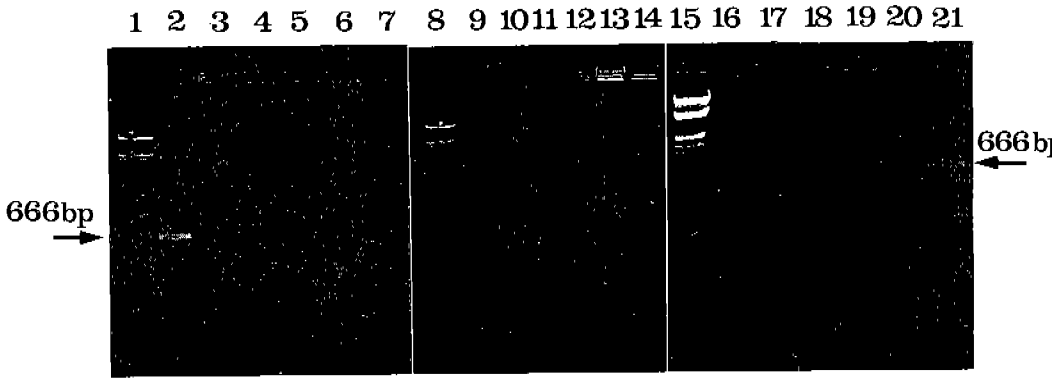

Fig. 2. PCR product with DNA of other organisms. The reaction parameters were same as those of Fig. 1. 1, size marker; 2, purified *P. carinii* DNA (W13-20); 3, *Escherichia coli*; 4, *Saccharomyces cerevisiae*; 5, *Entamoeba dispar*; 6, *Trichomonas vaginalis*; 7, *Leishmania major*; 8, size marker; 9, sparganum; 10, *Clonorchis sinensis*; 11, *Ascaris lumbricoides*; 12, *Gastroconger myriaster*; 13, rat; 14, human; 15, size marker; 16, *Streptococcus pneumoniae*; 17, *Neisseria sicca*; 18, *Staphylococcus aureus*; 19, *Candida albicans*; 20, sputum of a patient; 21, rat *P. carinii* (F19). Only *P. carinii* DNA from rats were positive and all with others were negative.

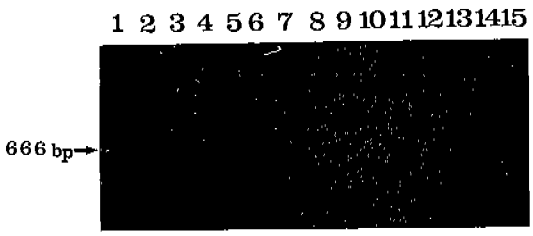

Fig. 3. PCR products separated in 0.7% agarose gel. Primers were #24 and #27, and PCR parameters were 94°C 2 min., 62°C 2 min., 74°C 2 min. in a thermal cycler. 1, lysate of an infected lung homogenate; 2-4, BAL fluid lysates from infected rats; 5-7, Sera from non-infected Wistar rats; 8-9, sera from infected Wistar rats; 10-15, blood from infected Sprague-Dawley rats.

100%. In reactions with serum, the sensitivity was 10% and the specificity was 100%.

DISCUSSION

PCR was introduced as a powerful technology which might solve the enigma of diagnosis of pneumocystosis (Kitada *et al.*, 1991; Sepkowitz *et al.*, 1993; Tamburrini *et al.*, 1993; Olsson *et al.*, 1993). Though the papers had presented excellent data, PCR is not used as a routine diagnostic method for clinical specimens in hospitals. The disadvantage of PCR diagnosis is that it is too tricky to make consistent products. The reaction varies greatly according to quality or quantity of prepared specimen DNAs, and also false positive reaction is frequent by contamination with previous products. Still sensitivity and specificity of morphological diagnosis is acceptable when BAL fluid is used.

The present study screened 5 sense primers and 4 antisense primers which were synthesized according to the known sequences of 16s rRNA. Among total 20 combinations of those primer pairs, the primer pair of #24 sense primer and #27 antisense primer gave the best product under the given reaction conditions. The primer pair produced no amplification with other organisms including DNAs of the rat or human. It is well-known that rDNA of *P. carinii* shares many homologic sequences with that of *Saccharomyces cerevisiae* or *Candida albicans* (Edman *et al.*, 1988; Stringer *et al.*, 1989). However, the primer pair of #24 and #27 showed no cross reaction with them. Also the primer pair produced no PCR amplification with the microorganisms which are common in the upper respiratory tract or oral mucosa. The primer pair is specific to *Pneumocystis carinii* so far.

As expected, the lysate of BAL fluid was found less sensitive than extracted DNA. The components in the lysis solution of *P. carinii* may inhibit the reaction. In the present study,

Table 2. Sensitivity and specificity of PCR amplification of rDNA with extracted DNAs and lysates of BAL fluid or serum

Prepared specimens	Microscopic examination	No. of cases by PCR			Sensitivity (%)	Specificity (%)
		Exam.	Positive	Negative		
BAL fluid	*P. carinii* +	26	15	11	57.7	
lysates	*P. carinii* -	13	0	13		100
DNA extracts	*P. carinii* +	26	22	4	84.6	
from BAL fluid	*P. carinii* -	13	0	13		100
Serum	*P. carinii* +	20	2	18	10.0	
	P. carinii -	4	0	4		100
Blood	*P. carinii* +	6	0	6	0	

the lysate was boiled to eliminate the activity of proteinase K, but the yield was still poor as 57.7%. The PCR sensitivity was improved to 84.6% after the DNA was extracted by phenol and chloroform purification. When any further PCR recipe whose sensitivity is acceptably high without the procedure of DNA extraction is developed, the PCR may be the standard method of diagnosis.

As for the diagnostic efficacy, PCR is well correlated with microscopic findings when the specificity of PCR is concerned. All of microscopically negative BAL fluids were negative by PCR and thus the specificity was 100% same as that of Cartwright *et al.* (1994).

The present data revealed that PCR could detect *P. carinii* in BAL fluid when the amount of DNA was about 10 fg. The amount is estimated as DNA of 10^5 organisms as the DNA of one nucleus of *P. carinii* is counted 0.1 pg and contamination by host DNA is neglected. However, the minimum number of *P. carinii* for PCR must be much less than 10^5 because much more host DNA is included in the prepared template DNA. When the cloned DNA of rRNA was applied to PCR, 0.1 fg DNA could make the product. This 1/100 fold difference means that the prepared DNA of BAL fluid or lung homogenate includes lots of host DNA. The host DNA included in template DNA of BAL fluid may hamper PCR if the contamination is considerably severe compared to the amount of *P. carinii* DNA. More host DNA than 100 ng in a reaction tube can inhibit the reaction (data not shown). The contamination amount of host DNA may be practically important because a few *P. carinii* are mixed with hundreds to thousands of host cells in most clinical specimens.

It would be a real progress if diagnosis of pneumocystosis were made by blood or serum. One paper suggested PCR with blood DNA of infected hosts should be a good non-invasive diagnostic measure (Kitada *et al.*, 1991). Also serum was supposed to be a good specimen for PCR amplification in diagnosis of pneumocystosis (Sepkowitz *et al.*, 1993). However serum or blood looks improper for PCR diagnosis of pneumocystosis. In the present study, only 2 infected sera were positive and none of infected blood was positive. As host phagocytic cells engulf *P. carinii*, the organisms may be transfered into the blood. Most of the phagocytosed *P. carinii* are degenerated rapidly after ingestion (Laursen *et al.*, 1993). When the DNA is released into serum, it is also rapidly degraded by endogenous nucleases. Since PCR is still possible as far as the target fragment is intact, PCR diagnosis of pneumocystosis with blood or serum DNA is possible but may be limited in consistency. The ratio of host DNA to *P. carinii* DNA is especially important for the consistency. Although a host is severely infected, the amount of *P. carinii* DNA in the blood or serum must be really negligible.

Most of the normal control rats were exposed to *P. carinii* during the experiment. Therefore they might harbour some organisms in their lungs and thus carry the DNA in their serum but the number or the amount was too small to be detected microscopically or by

PCR.

Conclusively, PCR would be a good diagnostic measure of pneumocystosis if non-invasive specimens were used and rapid and pure preparation of template DNA were available.

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=초록=

중합반응을 이용한 흰쥐 폐포자충증의 진단

홍성태

서울대학교 의과대학 기생충학교실 및 풍토병연구소

중합반응(PCR)은 극미량의 핵산을 찾아내어 소수의 감염병원체를 확인하는 매우 민감한 진단법이다. 폐포자충 같이 다수의 숙주세포에 소수의 병원체가 섞여 있는 가검물에서 핵산의 정제 여부에 따른 중합반응의 민감도를 관찰하고, 특이도가 높은 시발체(primer)를 개발하기 위하여 이 연구를 수행하였다. 흰쥐를 실험적으로 감염시키고, 폐, 폐포세척액, 혈청을 확보하여 현미경적 검사와 중합반응을 실시하였다. 또한 사람과 흰쥐의 핵산을 위시하여 여러 미생물과 기생충, 이스트의 핵산을 정제하여 이 시발체의 특이도를 검증하였다. 그 결과 여러 시발체 중에서 rRNA의 염기서열 중에서 선택한 #24 주서열과 #27 대서열 쌍이 가장 우수한 민감도와 특이도를 보였다. 형태학적으로 양성인 폐포세척액의 세포용해액으로 반응시킨 경우 민감도가 57.7%이며 핵산을 정제한 경우 84.6%로 증가하였다. 병원체 음성인 경우와 다른 병원체와 숙주의 핵산과는 반응하지 않았다. 혈청을 이용한 경우 20개 양성 표본 중 2개가 양성이고 6개의 감염된 흰쥐의 혈액은 모두 음성이었다. 중합반응을 폐포자충증의 진단에 활용하기 위하여는 폐포세척액 보다는 가래나 기관지 분비물, 혈청이나 혈액같은 비침습적인 가검물을 이용하고 핵산시료를 준비하는 과정이 간편하고 재현성이 있도록 개발되어야 할 것이다.

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