PCR Inhibition Assay for DNA-targeted Antibiotics

KUNIMOTO HOTTA*, CHUN-BAO ZHU†, POOLSAK PHOMSUWANSIRI†, JUN ISHIKAWA, SATOSHI MIZUNO, MASAHIRO HATSU[1] and SATOSHI IMAI[1]

Department of Bioactive Molecules, National Institute of Health, 1-23-1, Toyama, Shinjuku-ku, Tokyo 162, Japan
[1]Pharmaceutical Research Center, Meiji Seika Kaisha, Ltd., 760 Morooka-cho, Kohoku-ku, Yokohama 222, Japan

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DNA amplification by polymerase chain reaction (PCR) should be inhibited if the target for amplification region in the template DNA is nicked or cut. Based on this premise, we established a sensitive and differential assay using PCR to detect antibiotics that act on DNA. After template λDNA (10 pg) was incubated with antibiotics (10~20 ng) at 37°C for 30 minutes in a 5 μl reaction volume, a PCR assay (10 μl reaction volume; 25~30 cycles) was performed under the conditions we modified, resulting in amplification of a 500 bp fragment of λDNA which was monitored by agarose gel electrophoresis. Among the several antibiotics examined, the anthracyclines, bleomycin, D-cycloserine and mitomycin C clearly inhibited the PCR amplification reaction, whereas actinomycin D and ofloxacin did not. Preincubation of template DNA in the presence of Fe^{++} was necessary for bleomycin and cycloserine to exhibit marked inhibition of PCR. Mitomycin C exhibited the inhibition in the presence of DTT and Cu^{+}. By contrast, non-DNA-acting antibiotics (200 ng) such as aminoglycosides, β-lactams, and macrolides showed no inhibition. The PCR-amplified fragment from λDNA was not degraded by incubation with the antibiotics (20 ng) that inhibited PCR. Furthermore, ethylacetate extracts of the cultured broths of actinomycetes proved to be suitable as samples for this PCR inhibition assay.

Polymerase chain reaction (PCR)[1,2] has revolutionized gene technology and was developed for the *in vitro* amplification of specifically targeted DNA sequences. In principle, it is possible to amplify any specific region with known nucleotide sequences from even a single molecule of DNA. Basic requirements for PCR include a template DNA, two oligonucleotide primers corresponding to both ends of the target region, and a DNA polymerase such as Taq polymerase, respectively. However, if any one of these factors is damaged, the amplification of DNA will be inhibited. In this context, there are a variety of antibiotics that have been known to cause DNA strand scission. If such antibiotics make a nick or cut in the target for amplification of the template DNA, primer extension by DNA polymerase should stop at the nicked or cut site, resulting in total or strong inhibition of amplification of the target region. In addition, since an extremely low level of the template DNA is sufficient for DNA amplification by PCR, it seemed very likely that the level of antibiotics necessary for inhibition of PCR should be low.

Based on this insight, we attempted to establish a sensitive and differential PCR inhibition assay for antibiotics that are capable of nicking or cutting DNA strands. In practice, we did not use any specific template and primers, only the λDNA and primers provided as positive control materials as part of a commercial PCR kit, materials which are usually sitting in a freezer without any other specific use.

Materials and Methods

Polymerase Chain Reaction (PCR)

GeneAmp PCR Kit (Perkin Elmer Cetus), containing λDNA template, primers #1 and #2, dNTPs (dATP, dCTP, dGTP and dTTP) and Taq polymerase, was purchased from Takara Shuzo, Japan. The positive control conditions described for the amplification of a 500 bp fragment from λDNA were used as the standard conditions (Table 1). PCR (25 cycles unless otherwise mentioned) was carried out in a 0.5 ml plastic tube placed in a PCR incubator (Thermal Sequencer TSR-300: Iwaki Glass Co., Japan) under the following incubation conditions per PCR cycle: 95°C, 1 minute for DNA denaturation followed by 45°C, 1 minute for primer annealing and then 72°C, 1 minute for primer extension. To ensure good DNA denaturation in the first PCR cycle as well as complete DNA chain elongation in the last PCR cycle, both steps were carried out for 2 minutes.

† Present address: Shanghai Institute of Pharmaceutical Industry, 1320 Beijing Rd. (West), Shanghai, China (C. Z.) and Division of Drug Analysis, Department of Medical Sciences, Bangkok 10100, Thailand (P. P.).

After PCR was completed, the amplification of a 500 bp DNA fragment spanning nucleotide Nos. 7131~7160 of λDNA was monitored by subjecting 10 μl of the reaction mixture to agarose gel electrophoresis using 0.8% agarose ME (Marine Colloids, Div.) in TAE buffer containing 0.5 μg/ml of ethidium bromide[3].

For examination of the effect of antibiotics or ethylacetate extracts of cultured broths, these materials were incubated at 37°C for 30 minutes with the template DNA in a 5μl volume and then 5 μl of 2×PCR buffer containing double strength of primers, dNTPs and Taq polymerase was added in order to start PCR under the modified conditions described in Results.

Antibiotics

Antibiotics, except for ofloxacin which was a kind gift from Daiichi Pharmaceutical Co., were obtained as the preparation for injection from the Antibiotic Collection of National Institute of Health of Japan.

Ethylacetate Extraction of Cultured Broths of Actinomycetes

A portion (300 μl) of the cultured broths of actinomycetes was mixed with an equal volume of ethylacetate, vortexed for 30 seconds and centrifuged. Resulting supernatants were evaporated *in vacuo* to dryness and then resuspended with MilliQ water a volume equal to the supernatants. These solutions were diluted 10 fold for use in the PCR assay.

Results

Modification of PCR Conditions

To establish a PCR inhibition assay with a high sensitivity in a small reaction volume, the standard PCR conditions specified by the supplier (Table 1) were modified. First, we examined the effect of reaction volume on PCR using 10 pg/μl of λDNA as the template under the following incubation condition: 95°C/1 minute→45°C/1 minute→72°C/1 minute. As shown in Fig. 1-A, there was no difference in DNA amplification between 100 μl (standard volume) and 10 μl (smallest volume). Then, PCR using reduced concentrations of the template DNA (1 pg/μl) and dNTPs (0.4 mM) was attempted in 10 μl reaction volume. DNA amplification was clearly observed under these modified conditions (Fig. 1-B). Consequently, the modified conditions shown in Table 1 was used as the standard PCR conditions, hereafter.

Under the modified conditions, the use of *Streptomyces* DNA and primers for a 450 bp region of a cryptic kanamycin gene, *kan*[4], as substitutes for λDNA and primers #1 and #2, respectively, resulted in a good amplification of about 450 bp fragment as well (data not shown).

Effect of incubation time was also examined (Table 2). It turned out that DNA amplification was clearly observed even under the condition of 95°C/20 seconds→45°C/10 seconds→72°C/10 seconds, requiring a markedly reduced total incubation time (19 minutes). Although this condition saves time for completion of 25 cycles of PCR, the standard incubation condition requiring 77 minutes was used for examining the effect of antibiotics on PCR in the following experiments.

Effect of Antibiotics on PCR

Based on the above results, we designed the following procedure for the PCR inhibition assay. Template DNA (10 pg) was preincubated at 37°C for 30 minutes in the

Table 1. Standard and modified PCR conditions.

Constituent	Standard		Modified	
	Volume	Final concn.	Volume	Final concn.
H_2O	53.5 μl		5.95 μl	
10 x Reaction buffer[1]	10	1 x	1.0	1 x
dNTPs mixture[2]	16	200 μM	1.6	200 μM
Primer #1[3] (20μM)	5	1 μM	0.2	0.4 μM
Primer #2[3] (20μM)	5	1 μM	0.2	0.4 μM
λDNA[4]	10	10 pg/μl	1.0	1 pg/μl
Taq polymerase (5u/μl)	0.5	0.025 u/μl	0.05	0.025 u/μl
Total	100 μl		10 μl	
Oil	100 μl		15 μl	

1)500mM KCl, 100mM Tris·HCl pH8.3, 15mM $MgCl_2$, 0.1% gelatin.
2)1.25mM each of dATP, dCTP, dGTP and dTTP.
3)#1(25mer): 5'GATGAGTTCGTGTCCGTACAACTGG3'
#2(25mer): 5'GGTTATCGAAATCAGCCACAGCGCC3'
4)Concentration was 0.1 and 0.01ng/μl for standard and modified conditions, respectively.

Fig. 1. Amplification of λDNA fragment by PCR under varied conditions.

A: 25 cycles of PCR with 10 pg/μl λDNA. B: PCR with 1 pg/μl λDNA and 1 μM (a) or 0.4 μM (b) dNTPs.

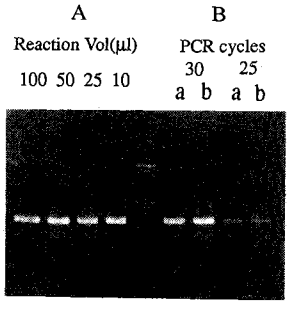
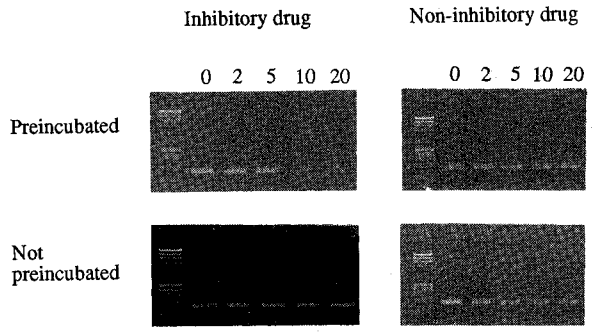


Table 2. Effect of incubation time on PCR.

Exp. No.	Incubation time (second) for PCR steps 95°C → (95°C → 45°C → 72°C) x 25 → 72°C					DNA amplification	Total time (min.)
1	60	60	60	60	60	YES	77
2	60	60	60	30	60	YES	64.5
3	60	60	30	30	60	YES	52
4	60	30	30	30	60	YES	39.5
5	60	20	20	20	60	YES	27
6	60	20	10	10	60	YES	18.7
7	60	10	10	10	60	NO	14.5
8	60	20	8	8	60	NO	17

Fig. 2. DNA amplification by PCR in the presence of antibiotics.



PCR was carried out with or without preincubation of λDNA (10 pg) with the indicated amount of antibiotics (ng).

presence or absence of antibiotics (2～200 ng) in a 5 μl reaction volume, and then 5 μl of 2×PCR buffer containing 400 μM dNTPs, 0.8 μM primers and 0.5 u of Taq polymerase was added in order to start PCR (25 cycles).

Fig. 2 represents the results obtained with an inhibitory antibiotic (bleomycin) and a non-inhibitory one (kanamycin); the former has been known to cause a

Table 3. Effect of antibiotics that interact with DNA on PCR.

Antibiotics	Amplification of lambda DNA					
	Preincubation			No Preincubation		
	0	10*	20*	0	10*	20*
None(control)	+++			+++		
Aclarubicin		+++	+++		+++	+++
Daunomycin		++	+		+++	+++
Doxorubicin		++	+		++	+
Epirubicin		++	+		++	+
Pirarubicin		−	−		+	−
Bleomycin**		−	−		+++	+++
Pepleomycin**		+	−		+++	+++
Actinomycin D		+++	+++		+++	+++
Chromomycin A_3		++	++		++	++
Cycloserine**		+++	−		+++	+++
Mitomycin C		++	+		++	++
Ofloxacin		+++	+++		+++	+++
5-Bromouracil		+++	+++		+++	+++
5-Fluorouracil		+++	+++		+++	+++
Acriflavine		++	+		++	+
Ethidium bromide		+	−		++	+

* ng of antibiotics incubated with template λDNA (10pg) prior to PCR. Symbols (+++ ~ -) refer to relative strength of amplified DNA bands to the control band. - refers to no band.
** These antibiotics were incubated with the template in the presence of equal molar concentrations of $FeSO_4$.

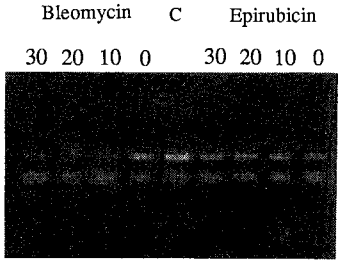
single strand scission of DNA in the presence of ferrous ion and molecular oxygen[5,6] and the latter to inhibit protein synthesis[7]. In comparison with the positive control (without antibiotic), bleomycin (10 ng/assay or higher in the presence of 1.3 μM $FeSO_4$) clearly inhibited amplification of a 500 bp fragment from λDNA template that was preincubated with the antibiotic. Substitution of *Streptomyces* DNA and *kan* gene primers for λDNA and primers #1 and #2 did not bring any change in the results (data not shown). However, no substantial inhibition was observed without preincubation. On the other hand, kanamycin did not inhibit the amplification even at 10-fold higher concentration (200 ng/assay) than bleomycin, regardless of preincubation conditions.

PCR results obtained with antibiotics that have been known to interact with DNA are summarized in Table 3. Clear inhibition of the DNA amplification was observed with anthracyclines (pirarubicin, and to a lesser extent, daunomycin, doxorubicin and epirubicin), bleomycin, peplomycin, cycloserine and mitomycin C when the template λDNA was preincubated with them. In addition, acriflavine, ethidium bromide which have been known to intercalate DNA[8] showed a similar inhibition. The PCR inhibition by bleomycin (Fig. 2), peplomycin and cycloserine occurred in the presence of a low level (1.3 μM) of Fe_2SO_4. Mitomycin C also inhibited PCR when preincubated with the template in the presence of 10 mM sodium hydrosulfite ($Na_2S_2O_4$)

and 1 mM $CuCl_2$ that are required for the single strand scission by mitomycin C of DNA[9].

Little or no clear inhibition was detected with aclarubicin (an anthracycline), actinomycin D[10] and chromomycin A_3[11] (inhibitors of DNA-dependent RNA synthesis) and ofloxacin (one of quinolone antibiotics that inhibit DNA gyrase[12]) at least under the concentrations tested.

Fig. 3. Effect of preincubation time of λDNA with antibiotics on PCR.



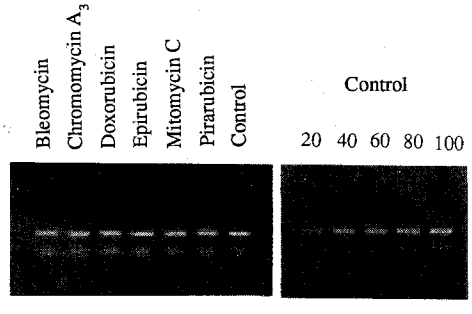
λDNA (10 pg) was incubated with antibiotics (10 ng) at 37°C for the indicated minutes.

Table 4. Effect of antibiotics that do not interact with DNA on PCR.

Antibiotics	Amplification of lambda DNA					
	Preincubation			No Preincubation		
	0	100*	200*	0	100*	200*
None(control)	+++			+++		
Ampicillin		+++	+++		+++	+++
Aztreonam		+++	+++		+++	+++
Carmonam		+++	+++		+++	+++
Cefoperazone		+++	+++		+++	+++
Penicillin G		++	++		++	++
Dibekacin		+++	+++		+++	+++
Fortimicin		+++	+++		+++	+++
Gentamicin		+++	+++		+++	+++
Kanamycin		+++	+++		+++	+++
Neomycin		+++	+++		+++	+++
Ribostamycin		+++	+++		+++	+++
Streptomycin		+++	+++		+++	+++
Chloramphenicol		+++	+++		+++	+++
Doxycycline		+++	+++		+++	+++
Leucomycin		+++	+++		+++	+++
Viomycin		+++	+++		+++	+++

* ng of antibiotics incubated with template λDNA (10pg) prior to PCR.

Fig. 4. Degradation test of PCR-amplified λDNA with antibiotics.



λDNA fragment (50 ng) amplified by PCR were incubated with antibiotics (20 ng) at 37°C for 30 minutes in 10 μl.

Fig. 3 shows the effect of preincubation time of the template with antibiotics on DNA amplification. Subsequently, it turned out that the preincubation was effective for bleomycin to exhibit clear PCR inhibition. However, there was no significant difference in PCR inhibition by epirubicin between the result with preincubation and the one without preincubation. Similar results were obtained with DNA-intercalating agents such as anthracyclines, acriflavine and ethidium bromide as seen in Table 3.

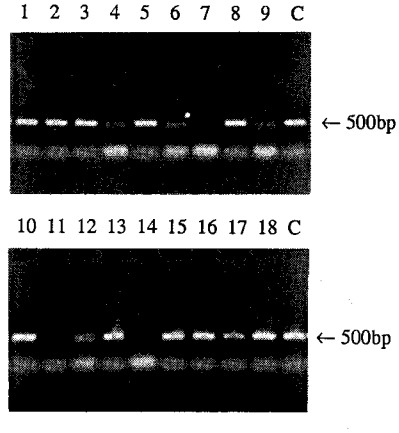
Table 4 shows the effect of a variety of antibiotics that cause no damage to DNA on the PCR assay. It turned out that none of them inhibited the DNA amplification by PCR even when the template DNA was exposed to 10-fold higher quantity (200 ng) of the antibiotics.

DNA degradation test was also carried out. The 500 bp λDNA PCR fragment (estimated at 50~100 ng) was incubated at 37°C for 30 minutes in 5 μl reaction volume with 20 ng of the antibiotics that inhibited PCR. However, no degradation was observed (Fig. 4). DNA degradation was observed when the same quantity of λDNA was exposed to 300 μg of bleomycin or adriamycin (doxorubicin).

Effect of Ethylacetate Extracts of Cultured Broths on PCR

The PCR inhibition assay established as above was applied for screening the cultured broths of actinomycetes for active substances. Two μl of centrifuged-supernatants (with or without heating at 70°C for 10 minutes) or ethylacetate extracts of about 250 cultured

Fig. 5. PCR in the presence of fermentation broth extract.



Ethylacetate extract samples (2 μl) were inucubated at room temperature for 10 minutes with 3 μl (10 pg) of λDNA prior to PCR.

broths were tested in the PCR inhibition assay. The supernatants exhibited a nonspecific inhibition in the PCR assay (data not shown). Selective inhibition was observed with the ethylacetate extracts as shown in Fig. 5. Of 18 samples shown, strong inhibition of the amplification of 500 bp λDNA fragment was observed with sample Nos. 7, 11 and 14, and to a lesser extent, with samples of 4, 6 and 9 compared to the positive control.

Discussion

Using PCR inhibition, antibiotics with activity of DNA strand scission could be selectively detected at very low concentrations. These antibiotics included DNA-intercalating agents (anthracyclines, acriflavine and ethidium bromide), and the bleomycin group antibiotics and cycloserine that have been known to cut DNA in the presence of ferrous ion and/or reducing agent. Mitomycin C also inhibited PCR when preincubated with the template in the presence of sodium hydrosulfite and Cu^{++} that are required for the single strand scission by the antibiotic of DNA[9]. Although we did not examined in this study, diyne-ene-containing antitumor antibiotics[12] such as neocarzinostatin[14], macromomycin[15], and esperamicins[16] including FR-900405[17] (=esperamicin A_{1b}) that are known to have 1,000 fold higher antitumor activity than doxorubicin (adriamycin) should also inhibit PCR.

Clear PCR inhibition was detected by the bleomycin group and cycloserine with addition of a low level of $FeSO_4$ and by anthracyclines without the addition of ferrous ion, although the reaction mixture of PCR inhibition assay was not supplemented with any reducing agent. This is due to the fact that the level of the template DNA was extremely low (1 ng/ml) compared to those levels (3~250 μg/ml) employed in the DNA degradation tests reported[5,18,19].

Preincubation is not necessary for DNA-intercalaters (anthracyclines, acriflavine and ethidium bromide) but is required for bleomycin to show PCR inhibitory activity. Since bleomycin does not require ferric ion (Fe^{+++}) but ferrous ion (Fe^{++}) for its DNA strand scission activity, the failure in PCR inhibition by bleomycin without the preincubation might be due to a rapid conversion of Fe^{++} to Fe^{+++} by exposing to the elevated high temperature (95°C, 2 minutes) upon initial heating for DNA denaturation in PCR.

Aclarubicin showed no significant activity in contrast with the other anthracylines. This will be due to its weak DNA breaking activity as reported in comparison with doxorubicin (adriamycin)[18].

For *in vitro* characterization of antibiotics that cause the scission or topological alteration of DNA, a variety of methods have been established. A popular one has been to examine the degradation or topological alteration of plasmid DNA by antibiotics. The levels of DNA and antibiotics were much higher in these systems than in the PCR inhibition assay we established. The level of the template DNA was extremely low (1 ng/ml) in the PCR inhibition assay, compared to those (3~250 μg/ml) employed in the DNA degradation tests reported[5,18,19]. Also the level of antibiotics necessary for DNA degradation is 40 μM in this plasmid assay, whereas only 4 μM is sufficient for the PCR inhibition assay in case of doxorubicin (adriamycin).

Thus, PCR inhibition assay can be regarded as a sensitive and differential assay for antibiotics with DNA-cutting activity. Thus compounds with weak DNA-cutting activity can be readily detected by the PCR inhibition assay. In this context, selective inhibition of PCR was observed with ethylacetate extracts of the cultured broth of actinomycetes. It seems thus likely that the PCR inhibition assay can be used for screening of novel compounds with DNA-cutting activity. In fact, we were successful in detecting PCR inhibition activity of a novel antimicrobial metabolite from an actinomycete strain (unpublished).

Recently, a unique PCR approach to evaluate antibiotic activity was reported by OHSHITA *et al.*[20,21]. They established a PCR method to determine the extent of gene-specific damage in human cells treated with cisplatin.

In this context, it seems that the PCR inhibition test could be a useful tool for assessing genotoxicity *in vitro*, perhaps in testing toxic waste. Alkylating agents might be detectable by this method by showing that DNA containing alkylated adducts can be subsequently cleaved by chemical or enzymatic means.

Our results suggest that not only PCR but also other *in vitro* enzymatic systems for the syntheses of cDNA (RT-PCR) as well as RNA can be the basis for establishing assay systems with high sensitivity and specificity.

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