韓國臨床獸醫學會誌(1995) 12(1): 117~122

Enhencement of Specificity of PCR Amplification of GC-rich *Mycobacterium paratuberculosis* DNA by Denaturants

Doo Kim[1], Yung-fu Chang*

Department of Veterinaty Medicine, College of Animal Agriculture, Kangwon National University, Chunchon, 200-162, Republic of Korea[1]
Diagnostic Laboratory, College of Veterinary Medicine*
Cornell University, Ithaca, NY, 14851-0786, USA

Denaturant에 의한 *Mycobacterium paratuberculosis* DNA의 PCR 증폭의 특이성 증진

김 두[1] · 창영후*

강원대학교 축산대학 수의학과
미국 코넬대학교 수의과대학 진단실험실*

요 약

GC 함유량(72%)이 높은 *Mycobacterium paratuberculosis*의 DNA의 PCR 증폭시 특이성과 생산성을 높이기 위하여 PCR 반응액에 denaturant인 DMSO, glycerol, formamide, Tween 20과 NP 40를 첨가하였다.

Denaturant를 첨가하지 않은 상태의 PCR에서는 다수의 비특이적인 DNA가 관찰되었으며 표적 DNA 생산량이 낮았다. 모든 denaturant는 PCR의 특이성과 생산물의 생산량을 증가시켰으며, 이들 중 DMSO, glycerol, formamide와 NP 40는 높은 농도에서 생산량을 증가시켰다. Tween 20은 낮은 농도에서 생산량을 증가시켰다.

Denaturant를 첨가하였음에도 불구하고 대부분의 반응에서 1 또는 2개의 비특이적인 DNA가 관찰되었다.

Key Words : PCR, Denaturants, *Mycobacterium paratuberculosis*, GC-rich DNA

[1] Correspondence

Introduction

The PCR is a very sensitive method commonly used in the emerging field of nucleic acid-based diagnostics[14]. PCR has been utilized with great success for the detection of infectious agents and identification of genetic mutations, as well as in the study and characterization of the human genome[14,15,17].

Since its initial introduction in 1985, PCR technology has advanced significantly. Use of a thermally stable DNA polymerase has allowed for enhanced specificity of reactions, as well as automation of the process[19].

Evolution of a variety of detection formats has transformed PCR from a research tool to a general method suitable for DNA diagnostics[14].

Because of the powerful amplification potential of PCR, multiple, so-called 'extra' bands are often seen in analytical gels in addition to the desired single band of amplified template; the multiple extra products are due to non-specific priming[12]. Complete denaturation of the template DNA is essential for the efficient annealing of primers to it while stable secondary structures in the template may interfere with primer extention [21]. If the annealing temperature is low, non-specific DNA fragments are amplified causing the appearance of the multiple extra bands. If the annealing temperature is too high, the hybrid between the template DNA and the primer may be unstable decreasing the yield of the desired product[18]. Optimization of PCR for any DNA template involves testing the denaturation, annealing temperatures and number of other variables.

Moreover GC-rich DNAs pose particular problems as PCR templates because of their high *Tm* values[22]. We wished to amplify a GC-rich(72 % GC) chromosomal DNA of *Mycobacterium paratuberculosis* which encodes for protein of 34KDa [11]. Many extra bands were observed and yields of PCR products were low in the preliminary experiments by PCR reaction buffer provided commercially. So, we determined a number of denaturants or co-solvents to define conditions for optimizing PCR amplification of this GC-rich DNA of *Mycobacterium paratuberculosis*.

Materials and Methods

Template DNA of *Mycobacterium paratuberculosis* ATCC 19689 was purified according to De Kesel's protocol[5].

Two oligodeoxyribonucleotide primers were synthesized by the phosphoramidite method using Model 394-08 DNA synthesizer(Applied Biosystems, USA). One was a 23-mer, 5'-AGGAGGATCCATGACCTACTCTC-3'; the other was a 21-mer, 5'-CACGTTGTCGACTAGGCGCGA-3'. Each PCR mixture contained 100 μl of a master mix consisting of four deoxynucleotide triphosphates(dATP, dCTP, dGTP, and dTTP), two primers, 5 U of Ampli*Taq* DNA polymerase(Perkin-Elmer Cetus, USA), reaction buffer for Ampli*Taq* DNA polymerase (10mM Tris-HCl pH8.3, 50mM KCl and 3mM $MgCl_2$), template DNA, and denaturants. The concentrations of eath denaturant added to PCR mixtures were such as; 1.2 %, 2.5 %, 5 % and 10 % DMSO; 5 %, 10 %, 15 % and 20 % glycerol; 1.2 %, 2.5 %, 5 % and 10 % formamide; 0.05 %, 0.1 %, 0.2 %, 0.4 % Tween 20 and 0.05 %, 0.1 %, 0.2 % and 0.4 % NP 40. Total volume was adjusted with destilled water. Each primer concentrations was 50 pmol. The cycling conditions in a Perkin-Elmer Gene Amp System 9600 thermal cycler were an initial incubation for 10 min at 94 ℃, followed by 35 cycles each of 30 s of denaturation at 94 ℃, 15 s of annealing at 45

℃, and 30 s of extention at 72 ℃. And then, the tubes were kept at 72 ℃ for 15 min. After reaction, 10 μl of each reaction mixture was electrophoresed on a 0.7 % agarose gel, and stained with ethidium bromide.

Results

A number of denaturants(DMSO, glycerol, formamide, Tween 20 and NP 40) were added to PCR reaction mixture to establish optimal conditions for amplificication of GC-rich chromosomal DNA of *Mycobacterium paratuberculosis*. The effect of different concentration of denaturants on the specificity of PCR amplification of GC-rich DNA is shown in Fig 1.

Effects of DMSO : DMSO concentrations up to 10 % eliminated most non-specific products and increased the efficiency of amplification of GC-rich DNA of *Mycobacterium paratuberculosis*, while lower concentrations of DMSO(1.2 % and 2.5 %) decreased the yields. DMSO eliminated most extra bands, while at least one extra band was seen yet.

Effects of Glycerol : Glycerol increased the specificity of PCR amplification at higher concentrations(15 % and 20 %), but lower concentrations(5 % and 10 %) were totally inhibitory, and the extra bands were seen at lower concentrations. The yields of PCR amplifications with glycerol were less than those with DMSO.

Effects of formamide : Formamide inhibited PCR amplification totally at lower concentrations (1.2 % and 2.5 %). At higher concentrations(5 % and 10 %), formamide increased the specificity of PCR amplification and extra band was not seen. The yields of PCR amplification were, however, lower than those of other detergents.

Effects of Tween 20 : Tween 20 improved the specificity and the yields of PCR amplification at higher concentrations(0.1 %, 0.2 % and 0.4 %), though the two extra bands were seen at higher concentrations. But PCR amplification was totally inhibited at concentration of 0.05 % Tween 20.

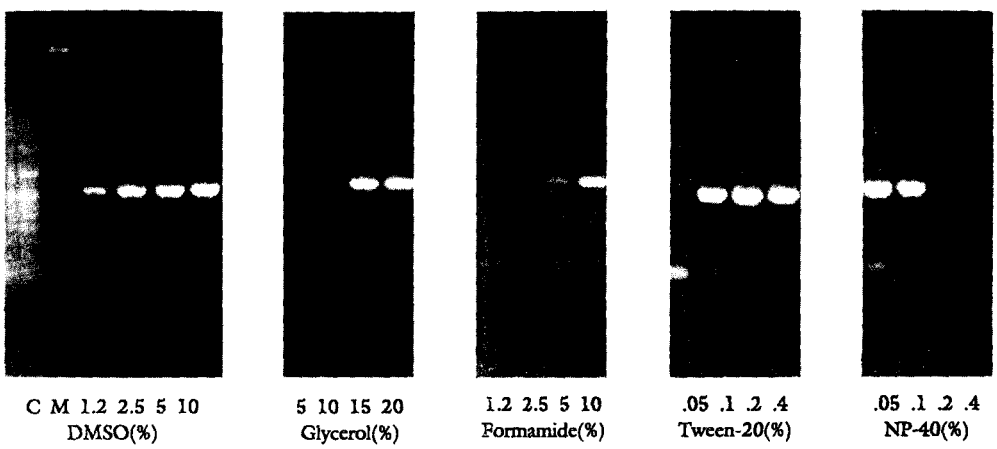

Fig 1. Effect of different concentration of denaturants on the specificity amplification of a GC-rich DNA of *Mycobacterium paratuberculosis*. The most intense band in each lane is at 948 bp. C, PCR product without denaturants; M, *Hind* Ⅲ digested λ DNA for molecular marker.

Effects of NP 40 : NP 40 was both specific and more efficient in its effects at lower concentrations(0.05 % and 0.1 %). The PCR amplification at concentration of 0.2 % NP 40 was very poor and PCR amplification was totally inhibited at 0.4 % NP 40.

Discussion

In general, organic solvents destabilize double-stranded DNA[13]. Solvents such as DMSO and formamide can paticipate in formation of hydrogen bonds and thereby reduce the formation of intramolecular hydrogen bonds in double-stranded DNA[7]. Although the specific mechanisms responsible for enhancement of DNA amplification by detergents or co-solvents are not known, it is possible that these agents may regulate the *Tm* values of primers and/or DNA templates by reducing the formation of intramolecular hydrogen bonds. Alternatively, they may decrease the heat sensitivity of the DNA polymerase[22].

DMSO and non-ionic detergents improve DNA sequencing reactions by decreaseing inter and intra strand reannealing[1,23]. DMSO was essential for amplification of the some genes[2]. With some templates, the presence of DMSO in low concentrations greatly improved priming specificity without affecting *Taq* DNA polymerase activity, especially when the primers were short and/or AT-rich; concentrations between 0.9 %~2 % eliminated misprimed products almost completely[6]. However DMSO at concentrations higher than 2 % inhibited DNA synthesis by Taq DNA polymerase[8]. But in this study, the higher concentrations of DMSO improved the productivity of PCR amplification than lower concentration.

Even glycerol inhibited amplication of some templates[21], glycerol increased the specificity of PCR amplification of certain DNA more significantly than did DMSO[16,21]. In our investigation, DMSO increased the specificity of PCR amplification and the yields more than did glycerol.

Formamide concentration up to 5 % eliminated most non-specific products and increased efficiency of amplification of G+C-rich(55 %) segments of the human dopamine D_2 receptor gene while 10 % formamide totally inhibited amplification. Specific amplification of the hla dqα gene with 5 % formamide has also been reported[3,20]. In our investigation, the higher concentrations of glycerol were more effective than the lower concentrations.

Non-ionic detergents such as Tween 20 and NP 40 reversed the inhibitory effect of 0.01 % SDS on *Taq* DNA polymerase[21]. Addition of 10 % DMSO or 0.5 % NP 40, or a combination of 0.5 % NP 40 with 0.5 % Tween 20 to DNA sequencing reaction mixtures enhanced specifity and the intensity of bands. With either NP 40 or combination of both non-ionic detergents, synthesis was greater than with DMSO[1], while, in our investigation, the lower concentrations of Tween 20 and the higher concentrations of NP 40 were more productive than DMSO, glycerol and formamide.

Mycobacterium paratuberculosis is a causative agent of Johne,s disease which affects a large proportion of ruminants in all continents and is the cause of important economic losses.

34KDa protein of *Mycobacterium paratuberculosis* contains B-cell epitopes-specific with respect to *Mycobacterium paratuberculosis*[10], and was applied to serological analysis of paratuberculosis[4]. In this study, we aimed to amplify DNA encoding 34KDa protein gene of *Mycobacterium paratuberculosis* for cloning work, and we could get right PCR products by addition of

above denaturants.

Conclusion

To establish optimal conditions for amplification of G+C-rich(72 %) chromosomal DNA of *Mycobacterium paratuberculosis*, we added a number of denaturants(DMSO, glycerol, formamide, Tween 20 and NP 40) to PCR reaction mixtures. In the absence of denaturants, amplified PCR products contained so many nonspecific bands and yields of expected specific band were very low. Addition of certain denaturants to PCR mixtures resulted in the improvement of the specificity and productivity of PCR. DMSO, glycerol, formamide and NP 40 increased the yield or production at high concentrations, on the other hand Tween 20 was more effective at lower concentration. Even in the presence of denaturants, the amlified PCR products still included one or two extra bands.

References

1. Bachmann B, Luke W, Hunsmann G. Improvement of PCR amplified DNA sequencing with the aid of detergents. Nucleic Acids Res 1990; 18: 1309.
2. Bookstein R, Lai CC, To H, Lee WH. PCR-based detection of a polymorphic BamH I site in intron 1 of the human retinoblastoma (RT) gene. Nucleic Acids Res 1990; 18: 1666.
3. Comey CT, Jung JM, Budowle B. Use of formamide to improve amplification of HLA DQα sequences. BioTechnique 1991; 10: 60-61.
4. De Kesel M, Gilot P, Coene M, Cocito C. Composition and immunological properties of the protein fraction of A36, a major antigen complex of *Mycobacterium paratuberculosis*. Scand J Immunol 1992; 36: 201-212.
5. De Kesel M, Gilot P, Misonne MC, Coene M, Cocito C. Cloning and expression of proteins of the 34-kilodalton protein gene of *Mycobacterium paratuberculosis*: Its application to serological analysis of Johne's disease. J Clin Microbiol 1993; 31: 947-954.
6. Filichkin SA, Gelvin SB. Effect of dimethyl sulfoxide concentration of specificity of primer matching in PCR. BioTechniques 1992; 12: 828-830.
7. Geiduschek EP, Herskovits TT. Nonaqueous solutions of DNA. Reversible and irreversible denaturation in methanol. Arch Biochem Bilophys 1961; 95: 114-129.
8. Gelfand DH. *Taq* DNA polymerase. In: PCR technology: Principles and applications for DNA amplification. New York: Stockton Press. 1989; 17-22.
9. Gelfand DH, White TJ. Thermostable DNA polymerase. In: PCR protocols. A guide to methods and applications. San Diego, CA:Academic Press. 1990; 129-141.
10. Gilot P, De Kesel M, Coene M, Cocito C. Induction of cellular immune reactions by A 36, an antigen complex of *Mycobacterium paratuberculosis*. Comparison of A36 and johnin components. Scand J Immunol 1992; 36: 811-821.
11. Gilot P, De Kesel M, Machtelincky L. Isolation and sequencing of the gene coding for an antigenic 34-kilodalton protein of *Mycobacterium paratuberculosis*. J Bacteriol, 1993; 175: 4930-4935.
12. Hung T, Mak K, Fong K. A specific enhancer for polymerase chain reaction. Nucleic Acids Res 1990; 18: 4953
13. Lee CH, Mizusawa H, Kakefuda T. Unwind-

ing of double stranded DNA helix by dehydration. Proc Natl Acad Sci USA 1981; 78: 2838-2842.

14. Loewy ZG, Baum HJ. Applications of molecular genetics for identity. In: Advances in genome biology. New York: JAI Press. 1993; 355-388.
15. Loewy ZG, Pottathil R. Polymerase chain reaction and its use in diagnostics. In: Diagnostics in the year 2000. New York: Van Nostrand Reinhold. 1993; 389-410.
16. Pomp D, Medrano JF. Organic solvents as facilitators of polymerase chain reaction. BioTechiques 1991; 10: 58-59.
17. Pottathil R, Loewy ZG. Aids diagnostics. In: Diagnostics in the year 2000. New York: Van nostrand Reinhold. 1993; 411-423.
18. Rychlik W, Spencer WJ, Rhoads RE. Optimization of the annealing temperature for DNA amplification in vitro. Nucleic Acids Res 1990; 18: 6409.
19. Saiki RK, Gelfand DH, Stoffel S, et al. Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science, 1988; 239: 487-491.
20. Sarkar G, Kapelner S, Sommer SS. Formamide dramatically improve the specificity of PCR. Nucleic Acids Res 1990; 18: 7465.
21. Smith TK, Long CM, Bowman B, Manos MM. Using cosolvents to enhance PCR amplification. Amplifications 1990; 5: 16-17.
22. Varadaraj K, Skinner DM. Denaturants or cosolvents improve the specificity of PCR amplification of a G + C-rich DNA using genetically engineered DNA polymerases. Gene 1994; 140: 1-5.
23. Winship PR. An improved method for directly sequencing PCR amplified material using dimethylsulfoxide. Nucleic Acids Res 1989; 17: 1266.