POSTER B272

The Inhibitors of Polymerase Chain Reaction in a Biological Culture

L. Zhang[1], B. Lam[2], Y. Haj-Ahmad[1,2]. [1]Brock University, St. Catharines, ON, CANADA. [2]Norgen Biotek Corp., Thorold, ON, CANADA

Abstract

Polymerase chain reaction (PCR) is widely used to detect the presence of pathogens in biological culture. In general, the detection limit of PCR is affected by the purity of the DNA samples. Impure substances from different sources might inhibit the PCR reactions and decrease the sensitivity. A comprehensive study was carried out to investigate the effect of PCR inhibitors on the amplification efficiency of DNA isolated from milk. PCR amplification was performed and compared using various amounts of DNA isolated from thirty raw milk samples by two different methods – a Proteinase K-based method and a silicon carbide column-based purification. Also, the effect of fat or lipids on the amplification efficiency was examined by amplifying the DNA samples prepared from milk with different fat percentages ranging from 0 to 10%. It was shown that the amplification efficiency was highly related to the amount of DNA isolated from the milk. In addition, a high fat to protein ratio in DNA samples resulted in low amplification efficiency. Lastly, we demonstrated that using an additional cleanup procedure with silicon carbide-based column effectively eliminates the inhibitors in milk

Introduction

• PCR as a milestone in modern biological research was invented in 1983 (1). PCR is a common technique used in medical and biological research labs for a variety of tasks, such as the sequencing of genes, the diagnosis of hereditary diseases, the identification of genetic fingerprints, the detection of infectious diseases, and the creation of transgenic organisms (2). However, a common technical problem with PCR is false negatives, often due to the presence of PCR inhibitors, which may interfere with cell lysis, degrade nucleic acid, or inactivate the thermo-stable DNA polymerase (1).

• PCR is a specific and sensitive method to diagnose pathogens in biological samples. However, false-positives, false-negatives or low amplification may be caused by inappropriate primers, improper time, incorrect temperature conditions, variable polymerase quality, PCR inhibitors or non-target DNA contamination in the biological culture. As suggested by Wilson (3) common inhibitors include various components of body fluids and reagents encountered in clinical and forensic science, food constituents, and environmental compounds. The inhibition of PCR is likely due to various physical and chemical elements interfering with the interaction between the templates of DNA and polymerase. Rossen et al. (1992) concluded that all of the most common inhibitory factors are elements such as organic and inorganic chemicals, detergents, antibiotics, buffers, enzymes, polysaccharides, fats, and protein in biological samples (4).

• In modern production, cow's milk is produced on an industrial scale. It is the most commonly consumed drink in the western world. Milk is synthesized in specialized cells of the mammary gland and is virtually sterile when secreted into the alveoli of the udder (2).

• PCR can supply more sensitive and reliable results than traditional methods. It has been shown that milk itself can serve as a useful source of DNA for PCR, and recently this technique has been applied successfully to control for authenticity of dairy products and to differentiate between species relevant to the dairy industry (5). However, milk is a special biological sample, because of the high amounts of protein, calcium ions and fats in milk samples which will interfere with preparing a pure DNA sample that is suitable for PCR amplification. In cow's milk, the somatic cells must be higher than bacterial flora, so the low target DNA will cause problems for detection of bacterial species.

• The objective of this study was to investigate the effect of PCR inhibitors on the amplification efficiency of DNA isolated from milk, using a previously reported method as well as a commercially available method. Efforts were also made to determine the type of PCR inhibitors present in milk samples.

Methods

Milk DNA Extraction
Milk DNA was isolated from 30 raw milk samples by two different methods; using Norgen's Milk Bacterial DNA Isolation Kit and the Proteinase K / phenol-chloroform method (7). The milk DNA samples were stored at -20°C.

PCR of Plasmid pCMVβ in the Presence of Milk DNA Extraction
The pCMVβ plasmid was amplified in the presence of variable amounts of milk DNA prepared using Norgen's Milk Bacterial DNA Isolation Kit or the Proteinase K / phenol-chloroform method with the following primers:
Forward primers (sequence: CGCTACCATTACCAGTTG)
Rreverse primers (sequence: TTAGCTCACTCATTAGGCA)

Additional Milk DNA Purification
Milk DNA that was prepared by the Proteinase K / phenol-chloroform method was further purified using a silicon-carbide column (Norgen's PCR Purification Kit). Different numbers of washes and types of washes were investigated to purify the DNA.

Protein Isolated from Different Fat Percentages of Commercial Milk
Milk proteins were isolated from 0%, 1%, 2%, 3.25% and 5% fat commercial milk using Norgen's ProteoSpin™ Urine Protein Concentration Micro Kit. The protein concentration was determined by the Bradford Protein Assay.

Gel Electrophoresis of PCR Amplification Products
Agarose gels were made by dissolving 1% (w/v) agarose into TAE buffer, and adding 8 µL of 0.5 µg/mL ethidium bromide. 20 µL of PCR products and 5 µL of 6X loading dye were loaded into the wells. The agarose gels were run at 160 V, and the gels were visualized using the Alpha Innotech AlphaImager 2200.

Results

Table 1. Amplification Results when a Non-Related PCR Reaction is Spiked with Various Amounts of Milk DNA Isolated Using 2 Different Methods

Volume of Milk DNA	Proteinase K / Phenol-Chloroform Method (Total 30 samples)	Norgen's Milk Bacterial Isolation kit (Total 30 samples)
1 µL	27 amplified (90 %)	28 amplified (93 %)
2 µL	20 amplified (67 %)	27 amplified (90 %)
3 µL	17 amplified (57 %)	23 amplified (77 %)
4 µL	13 amplified (43 %)	23 amplified (77 %)
5 µL	11 amplified (37 %)	20 amplified (67 %)
10 µL	0 amplified (0 %)	4 amplified (13 %)

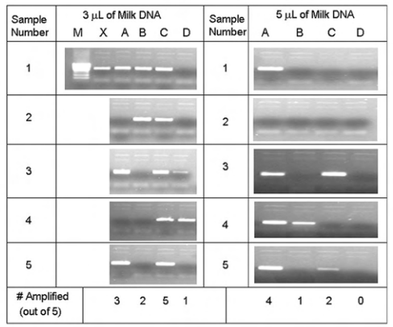
To generate the data in Table 1, DNA was isolated from 30 raw milk samples using two methods; Proteinase K / phenol-chloroform extraction and Norgen's Milk Bacterial DNA Isolation Kit. Various amounts of the isolated DNA were used to spike a 20 µL PCR reaction where the amplicon is not dependent on the amount of bacterial or bovine gDNA present. Over 90% of samples using 1 or 2 µL of DNA prepared by silicon-carbide column-based isolation (Norgen's kit) did not show any PCR inhibition. Over 60% of samples still showed no inhibition at up to 5 µL of DNA spiking. On the other hand, for the proteinase K / Phenol-chloroform method, the percentage of samples that showed no PCR inhibition drastically decreased when more than 2 µL of DNA was used. Less than 50% of samples showed no inhibition when more than 5 µL was used

Table 2. Amplification Results when a Non-Related PCR Reaction is Spiked with Various Amounts of Milk DNA Isolated Using the Proteinase K / Phenol-Chlorofom Method Follwed by Purification on a Silicon-Carbide Column using Either One or Two Washes

Amount of Purified Milk DNA	One Wash	Two Washes
1 µL	Good amplifications 29/30 amplified (97 %)	
5 µL	Good amplifications 22/30 amplified (73 %)	Good amplifications 29/30 amplified (97 %)
10 µL	No amplification 0/30 amplified (0 %)	Poor amplifications 4/30 amplified (13 %)

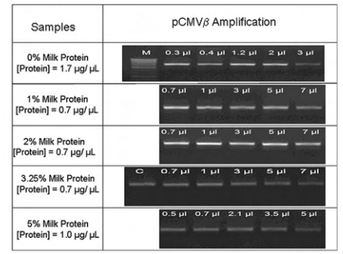
To generate the data in Table 2, milk DNA isolated using the Proteinase K / phenol-chloroform extraction method was further purified using silicon carbide-based columns to eliminate the previously observed inhibitors. DNA was purified using Norgen's PCR Cleanup Kit with one or two washes. Varying amounts of the purified milk DNA were again used to spike a non-related PCR. Interestingly, the quality of the DNA improved dramatically. All but one of the original 30 samples showed good PCR amplification results when 1 µL was used for spiking after only one wash. Moreover, PCR efficiency in reactions spiked with 5 µL of purified DNA increased from 37% to 73% after one wash, and this was further increased to 97% after two washes.

Table 3. Amplification Results when a Non-Related PCR Reaction is Spiked with Various Amounts of Milk DNA Isolated Using the Proteinase K / Phenol-Chlorofom Method Follwed by Purification on a Silicon-Carbide Column using 4 Different Washes



In order to identify the type of PCR inhibitor present, the original inhibitory milk DNA isolated by the Proteinase K / phenol-chloroform method was subjected to cleanup on silicon carbide-based column using various wash solutions. The four different Wash Solution are A (0.5 M EDTA, pH 8.0), B (10 % SDS), C (70 % ethanol) and D (distilled water). Either 3 µL or 5 µL of the purified and washed milk DNA was then used to spike a PCR amplification reaction. The control (X) is template DNA that has not been spiked. From observing Table 3 it can be seen that Wash A (0.5 M EDTA, pH 8.0) and Wash C (70% ethanol) were the most successful at removing inhibitors and allowing for successful PCR amplification. These results suggest that the inhibitors may be of organic origin or are divalent ions.

Table 4. pCMVβ Amplification in the Presence of Proteins Isolated from Different Fat Percentages of Commercial Milk



Since washing the milk DNA with ethanol was shown to be effective to remove PCR inhibition, one possible hypothesis was that proteins present in milk may be co-purifing with DNA and resulting in PCR inhibition. In order to determine whether milk proteins affect PCR, proteins were isolated from various types of commercially available milk using a silicon carbide-based protein extraction kit. The isolated proteins were used to spike PCR reactions. As the protein amount increased the amplified bands show less intensity (more inhibition) at the same fat percentage of milk. Under the same protein concentration, the protein isolated form high fat percentage milk show more inhibition than low fat percentage. Therefore, both proteins and fat are inhibitors of PCR amplification.

Conclusions

• Milk DNA prepared by the commercial kit (Norgen's Milk Bacterial DNA Isolation Kit) shows less inhibition than DNA isolated using the Proteinase K / phenol-chloroform method (Table 1).

• As the amount of inhibitors (milk DNA prepared by both methods) increased from 1 µL to 10 µL, the amplification of pCMVβ decreased. (Table 1). The decrease was greater for PCR reactions spiked with Proteinase K / phenol-chlorofom isolated milk DNA.

• Milk DNA isolated using the Proteinase K / phenol-chloroform method could be further purified using a silicon-carbide based column ` and 1 or 2 washes (Norgen's PCR Purification Kit). The DNA further purified using 1 wash showed much less PCR inhibition compared to DNA that was not further purified. Additionally, DNA that was further purified using 2 washes showed less PCR inhibition than the DNA that was further purified using 1 wash (Table 2).

• Silicon carbide-based columns can efficiently remove the PCR inhibitors in milk DNA

• Washes with EDTA and 70% ethanol were most effective at removing the inhibitors, suggesting they are organic compounds or divalent ions (Table 3)

• As the protein amount increased, the amplified bands show less intensity (more inhibition) at the same milk fat percentage. For the same protein concentration, proteins isolated from high fat percentage milk showed more inhibition than low fat percentage (Table 4).

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Contact Information:
3430 Schmon Parkway
Thorold, ON CANADA
L2V 4Y6
Phone: (905) 227-8848
Fax: (905) 227-1061
yousef@brocku.ca