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A Comparison Between Cerebrospinal Fluid Pretreatment Protocols Prior to Diagnosis of Herpes Simplex Encephalitis by PCR

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Abstract: In the present study the detection of HSV genome in the CSF by the Polymerase Chain Reaction (PCR) has been shown to be a promising tool in the early diagnosis of HSE. However in HSE the CSF may contain certain amounts of haem and protein which may inhibit the activity of the enzyme Taq polymerase (responsible for DNA copying). In addition when PCR is used for rapid diagnosis of HSE, the DNA extraction method (prior to PCR) should be rapid, simple and robust. This project was designed to evaluate a number of DNA isolation and purification protocols. Hence normal CSF was designed to evaluate a number of DNA isolation and purification protocols. Hence normal CSF was spiked with varying amount of HSV DNA followed by subsequent testing by PCR. In this investigation one of the protocols (phenol/chloroform + ethanol precipitation) showed 100% sensitivity of detection while others varied from 0 to 80% of sensitivity.

Key words: Herpes simplex virus encephalitis, DNA extraction, PCR

INTRODUCTION

Herpes Simplex Encephalitis (HSE) represents one of the most severe infectious diseases of the Central Nervous System (CNS). Treatment with the antiviral drugs such as: acyclovir and fuscarnet have been shown to significantly reduce both the mortality and morbidity of the disease (Klapper and Cleator, 1997; Skaldenberg *et al*., 1984) if provided treatment is initiated at early stage of the disease. Therefore rapid diagnosis of the disease is crucial (Puchhammer-Stõckle *et al*., 1990).

In recent years the Polymerase Chain Reaction (PCR) has become available for the specific and highly sensitive detection of nucleic acids (Saiki *et al*., 1988). PCR enables the detection of Herpes Simplex Virus (HSV) specific DNA in lumbar Cerebrospinal Fluid (CSF) and provides a powerful technique for the early diagnosis of HSE (Rowley *et al*., 1990; Aureluis *et al*., 1991; Pohl-Koppe *et al*., 1992; Puchhammer-Stõckle *et al*., 1993). However the activity of taq polymerase (the enzyme responsible for DNA amplification in PCR) can be inhibited by several substances including protein and haem (Aureluis *et al*., 1991; Powel *et al*., 1990). Hence direct addition of lumbar CSF to the reaction mixture can be used, however because there is a possibility of activities by inhibitors, false negative reactions may occur. There have been several publications describing methods for pretreatment of CSF, but each has certain limitations with respect to efficiency of recovery of DNA, time required for processing, technical complexity and so on. Nevertheless no comparison of the sensitivity of these and other methods that might be used to extract DNA from CSF has been published. This project was designed to investigate several pretreatment protocols to compare the efficiency of DNA recovery plus assessment of their advantages and disadvantages.

MATERIALS AND METHODS

Normal CSF: It was obtained from patients without signs of neurological disease, the CSF contained normal (<0.45 g dL^{-1}) total protein and no red or white blood cells on microscopic examination were seen.

Spiking of CSF with viral DNA: Normal CSF was spiked with viral DNA so that it contained: 50 pfu/50 µL of CSF; 25 pfu/50 µL of CSF; 10 pfu/50 µL of CSF; 5 pfu/50 µL of CSF and 1 pfu/50 µL of CSF.

Positive controls: By diluting viral DNA in sterile distilled water ($S.D.H_2O$) to contain: 50 pfu/10 µL of $S.D.H_2O$; 25 pfu/10 µL $S.D.H_2O$; 10 pfu/10 µL of $S.D.H_2O$; 5 pfu/10 µL of $S.D.H_2O$ and 1 pfu/10 µL of $S.D.H_2O$.

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These positive controls were once analyzed by PCR to make sure that they were detected by PCR (Fig. 1).

Negative controls: They were prepared by aliquots of 50 μL of Vero cells DNA and were isolated exactly in the same way that CSF samples were pretreated.

Contamination controls: For this purpose S.D.H_2O was used.

Primers: The oligonucleotide primers were supplied dissolved in 1 ml of S.D.H_2O and their sequences were:

Nest 7: 5' - CGC GCG GTA CCT TAT GGG CAG CAT GA-3'
Nest 8C: 5' - CAG GGT AAA TAA CGT GTC CCC GAT ATG G-3'

PCR reaction mixture: A single PCR reaction mixture (PCR cocktail) was composed of: 53.5 μL of S.D.H_2O, 16 μL of dNTP's mixture, 10 μL of PCR reaction mixture (1 mL 1M Tris-HCl, 5 mL 1M KCl, 200 μL 1M $MgCl_2$, 1 mL 2% W/V of gelatin and 2.8 mL of S.D.H_2O), 5 μL of primer 1 (nest 7) at 1:100, 5 μL of primer 2 (nest 8C) at 1:100, 0.5 μL of amplitaq equal to 2.5 units.

Addition of DNA to reaction mixture: When reaction mixtures were prepared 10 μL of extracted DNA was added to cocktail.

Extraction of DNA from CSF samples: Eight distinct protocols were chosen for DNA extraction from spiked CSF samples containing from 1 to 50 pfu as stated earlier. Protocol 1 was designed by Klapper *et al.* (1990); in which to 50 μL of CSF 350 μL of 1xTE buffer (containing 100 mL 1M Tris HCl, pH: 8, 20 mL 0.5 M EDTA pH:8 and 880 mL of S.D.H_2O) plus 400 μL of phenol/chloroform were added. The reagents were mixed and centrifuged at 12000 g for 2 min. The upper aqueous layer was collected and transferred to an eppendorf. The above steps were repeated except that only chloroform was used. Subsequently to the upper aqueous layer 40 μL of 3M Na acetate PH: 5.2 and 800 μL of absolute alcohol was added and incubated at -20°C for an hour. The mixture was then centrifuged at 12000 g for 5 min. The supernatant was removed and pellet left at room temperature to air dry for 5 min. The dried pellet was resuspended in 20 μL of S.D.H_2O. DNA was measured by UV spectrophotometer at 260 and 280 wave length versus the blank which was S.D.H_2O.

N.B: pure DNA gives a ratio of $^{O.D260}/_{O.D280}$ of 1.8

Protocol 2 was proposed by Boom *et al.* (1990), in this method to 50 μL of CSF, 900 μL of lysis buffer (2 mL Tris HCl PH: 8.3, 0.74 g KCl, 0.9 mL Nanidet P40, 0.9 mL Tween 20 and 0.1 g $MgCl_2$ to final volume of 100 mL with distilled water) and 40 μL of silica particles were added. The mixture was vortexed and incubated at room temperature for 10 min. After another vortexing of the mixture was centrifuged at 12000 g for 15 sec. Subsequently the supernatant was discarded and the pellet was washed twice with 6M guanidine thiocyanate. The pellet was again washed twice with 70% ethanol and once with acetone. The pellet was incubated at 56°C for 10 min to dry and to the dried product 50 μL of S.D.H_2O was added followed by vortexing and incubating at 56°C for 10 min. The suspension centrifuged at 12000 g for 2 min and in final step the supernatant was collected and O.D was measured as in previous protocol.

The next protocol (No. 3) was designed by Grimberg *et al.* (1989)according to this procedure to 50 μL of CSF 50 μL of PLB buffer (2 mL 1M Tris HCl pH :8.0, 4 mL 0.5M EDTA, 0.01 g NaCl, the final volume adjusted to 100 mL with S.D.H_2O) and to this mixture 5 μL of proteinase K (20 mg mL^{-1}) was added and incubated at 56°C for 2 h. The whole mixture was centrifuged at 12000 g for 1 min and the supernatant was retained for O.D measurement.

In case of protocol 4 (Brice *et al.*, 1989) once more to 50 μL of CSF 350 μL of S.D.H_2O was added followed by addition of 50 μL proteinase K to the mixture in an eppendorf. The plastic tube was incubated at 56°C for 2 h, subsequently the aliquot was incubated at 95°C for 10 min. The mixture was microcentrifuged at 12000 g for 1 min, the supernatant was then retained for O.D measurement of DNA as in previous protocols.

In 5th protocol proposed by Rowley *et al.* (1990) to 50 μL of CSF, 150 μL of S.D.H_2O and 200 μL of lysis buffer was added followed by addition of 1.2 μL of proteinase K in an eppendorf. The whole mixture was incubated at 56°C for 1 h. Furthermore 400 μL of phenol/chloroform was added to the aliquot and after microcentrifugation at 12000 g for 2 min the aqueous and organic phases were separated. The upper aqueous layer was transferred to a sterile eppendorf followed by repetition of above mentioned steps except that only chloroform was used. Once more to the upper aqueous layer 40 μL of 3M Na acetate pH: 5.2 and 800 μL of absolute alcohol were added, mixed and incubated at -20°C for an hour followed by microcentrifugation of the tube at 12000 g for 20 min. The supernatant was removed and the pellet was left at room temperature to air dry for 5 min. Twenty microliter of S.D.H_2O was added to the dried pellet for resuspension of DNA that was measured as previous protocols.

The 6th protocol was designed by Shibata *et al.* (1988), as a result to 50 μL of CSF, 350 μL of S.D.H_2O was

added and the mixture was incubated at 95°C for 10 min. The eppendorf was removed and cooled down at room temperature and to the mixture 40 μL of 3M Na acetate pH:5.2 and 800 μL of absolute alcohol were added. The whole mixture was then incubated at -20°C for 1 h followed by microcentrifugation at 12000 g for 20 min. When the supernatant was removed the pellet was left at room temperature to air dry for 5 min and resuspended in 20 μL of S.D.H_2O and O.D was measured as before.

The next protocol applied for CSF pretreatment was published by Lench *et al.* (1988). In this technique to 50 μL of CSF, 350 μL of S.D.H_2O was added and the mixture was then placed in boiling water bath for 10 min followed by microcentrifugation of tube at 12000 g for 30 sec. The supernatant was removed and O.D was measured by making a dilution of 1:100 in S.D.H_2O.

The final protocol employed was proposed by Harding *et al.* (1989). For this protocol DNA capture reagent kit was used instead of chemical reagents. Hence to 50 μL of CSF, 40 μL of extraction buffer and 10 μL of proteinase K solution were added. The mixture incubated at 56°C for 1 h, after vigorous vortexing of DNA capture reagent, 50 μL of slurry was added to the mixture and it was again vortexed and left on a rotator at room temperature for 2 h. To the combination 100 μL of TNE buffer added, vortexed, washed, centrifuged and discarded the supernatant. The latter step was repeated twice and to this mixture 50 μL of 0.1M KOH was added and the plastic tube was vortexed for 15 sec, left on a rotator for 10 min at room temperature. After microcentrifugation and collection of supernatant which contained DNA, 25 μL of 7.5M ammonium acetate and 200 μL of ethanol were added. The eppendorf was again microcentrifuged and the supernatant was removed and the pellet was resuspended in 20 μL of S.D.H_2O and O.D measured as before.

For each protocol spiked CSF samples containing 50, 25, 10, 5 and 1 pfu HSV DNA per 50 μL and 50 μL of Vero cells DNA as negative control were processed along with positive control and contamination control. Ten microliter of extracted DNA was added o PCR reaction mixture and it was overlaid with few drops of liquid paraffin.

Thermocycling of mixture: Thermocycler was programmed according to following data: 94°C for 7 min; 50°C for 1.5 min; 70°C for 2 min; 94°C for 2 min (steps 2, 3 and 4 were repeated for 50 times); 50°C for 1.5 min; 70°C for 2 min; 35°C for 4 min and refrigeration.

After thermocycling 15 μL of cocktail plus 1.5 μL of loading buffer pipetted into an eppendorf.

Preparation of agarose gel: A 1.5% (W/V) agarose gel in 1X TAE buffer was prepared and poured into a carrier and left to solidify. The gel was loaded with 16.5 μL of mixture of cocktail and loading buffer per well versus 7 μL of 1 kb ladder into the first well. In case of agarose electrophoresis, a constant voltage of 50 V was applied for about 2 h.

Staining the gel: For this ethidium bromide (10 mg mL^{-1}) and 1X TAE buffer (final volume 1 μL mL^{-1}) was applied for 10-15 min followed by destaining the gel in S.D.H_2O. The final step was U.V transillumination of the gel, searching for visible bands.

RESULTS

The location of specifically amplified band, which is 350 bp and comprises a portion of the virus specified for enzyme Thymidine Kinase (TK). The molecular weight of the amplified fragment is determined by inclusion of a 1 kb ladder.

Viral DNA Directed amplification (no pretreatment): In Fig. 1 viral DNA Directed amplification (no pretreatment) was showed. The aim of this amplification was to check purity of viral DNA and sensitivity of PCR. The bands did prove purity of viral DNA as well as accuracy of the PCR even with the least amount of DNA which was only 1pfu/10 μL.

Analysis of spiked CSF by PCR: Spiked CSF samples without any pretreatment were introduced directly onto PCR. The aim was to determine whether CSF was

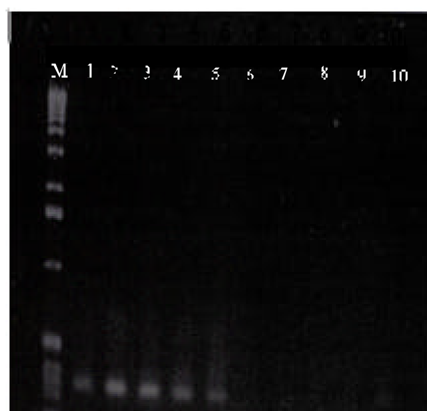

Fig. 1: Viral DNA direct amplification without pretreatment.
Lane 1: Untreated DNA 50 pfu/10 μL of S.D.H_2O.
Lane 2: Untreated DNA 25 pfu/10 μL of S.D.H_2O.
Lane 3: Untreated DNA 10 pfu/10 μL of S.D.H_2O.
Lane 4: Untreated DNA 5 pfu/10 μL of S.D.H_2O.
Lane 5: Untreated DNA 1 pfu/10 μL of S.D.H_2O.
Lane 6: S.D.H_2O

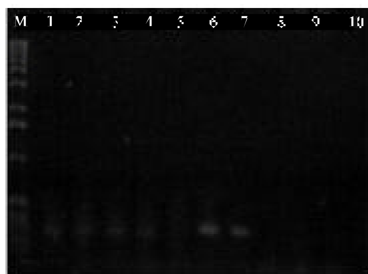

Fig. 2: Analysis of spiked CSF by PCR. Spiked CSF samples without any pretreatment were introduced onto PCR. Lane 1: Untreated CSF 50 pfu/50 μL of CSF. Lane 2: Untreated CSF 25 pfu/50 μL of CSF. Lane 3: Untreated CSF 15 pfu/50 μL of CSF. Lane 4: Untreated CSF 5 pfu/50 μL of CSF. Lane 5: Untreated CSF 1 pfu/50 μL of CSF. Lane 6: Positive control 50 pfu/50 μL of S.D.H_2O. Lane 7: Positive control 25 pfu/50 μL of S.D.H_2O. Lane 8 and 9: Negative control Vero DNA. Lane 10: S.D.H_2O

inhibitory to PCR since, untreated CSF contain variable amount of protein, which is an obstacle to taq polymerase activity. This experiment was carried out versus positive controls as shown in Fig. 2 the untreated CSF possessed inhibitory property.

Analysis of the samples by PCR: Pretreatment according to protocols designed by Klapper *et al.* (1990) (this is our standard protocol which is a modification of Maniatis DNA extraction method) Boom *et al.* (1990) and Grimberg *et al.* (1989) 1 to 3, respectively. The results are shown in Fig. 3 indicated that, the only method with maximum acuity was standard protocol while other procedures failed to extract viral DNA partially or totally.

The next pretreatment protocols applied in this project were designed by Brice *et al.* (1989) (No. 4) and Rowley *et al.* (1990) (No. 5). The latter procedure was time-consuming and laborious so, possibility of mishandling is inevitable. We repeated the experiment several times and continuously observed that DNA extraction was not reproducible even at high pfu as shown in Fig. 4.

Pretreatment according to protocols designed by Shibata *et al.* (1988) and Lench *et al.* (1988) (6 and 7, respectively) different steps were explained in detail in materials and methods section. We also noticed that these methods are not suitable for DNA extraction from CSF due to discrepancies as shown in Fig. 5.

The final protocol applied was the one proposed by Harding *et al.* (1989 (protocol 8) that employed a type of commercial kit as shown in Fig. 5. The same findings were also observed in case of the final procedure as former protocols with discrepancies and similar justifications can be acknowledged here.

Fig. 3: Pretreatment were done according to Klapper *et al.* (1990), Boom *et al.* (1990), Grimberg *et al.* (1989) (protocol 1, 2 and 3, respectively). Protocol 1: Lane 1: 50 pfu/50 μL of CSF (protocol 1). Lane 2: 25 pfu/50 μL of CSF. Lane 3: 10 pfu/50 μL of CSF. Lane 4: 5 pfu/50 μL of CSF. Lane 5: 1pfu/50 μL of CSF. Protocol 2: Lane 6: 50pfu/50 μL of CSF. Lane 7: 25 pfu/50 μL of CSF. Lane 8: 10 pfu/50 μL of CSF. Lane 9: 5 pfu/50 μL of CSF. Lane 10: 1 pfu/50 μL of CSF. Protocol 3: Lane 11: 50 pfu/50 μL of CSF. Lane 12: 25 pfu/50 μL of CSF. Lane 13: 10 pfu/50 μL of CSF. Lane 14: 5 pfu/50 μL of CSF. Lane 15: 1 pfu/50 μL of CSF. Lane 16: +ve control 50 pfu/10 μL of S.D.H_2O. Lane 17: +ve control 25 pfu/10μL of S.D.H_2O. Lane 18: -ve control (Vero DNA pretreated by protocol 2). Lane 19: -ve control (Vero DNA pretreated by protocol 3). Lane 20: Contamination control (S.D.H_2O). Lanes 1-8, 10, 16 and 17 were positive

DISCUSSION

Herpes Simplex Encephalitis (HSE) still carries a high morbidity and mortality, if treatment is commenced early, it can decrease high mortality rate associated with this disease. A common procedure for early detection of HSV genome in the CSF is the Polymerase Chain Reaction (PCR) in order to accompolish this technique DNA extraction plays a critical role.

Fig. 4: Lane 1: +ve control 50 pfu/10 µL of S.D.H_2O.
Lane 2: +ve control 25 pfu/10 µL of S.D.H_2O.
Lane 3: +ve control 10 pfu/10 µL of S.D.H_2O.
Lane 4 +ve control 5 pfu/10 µL of S.D.H_2O.
Lane 5: +ve control 1 pfu/10 µL of S.D.H_2O.
Lane 6: 50 pfu/50 µL of CSF (protocol 4).
Lane 7: 25 pfu/50 µL of CSF (protocol 4).
Lane 8: 10 pfu/50 µL of CSF (protocol 4).
Lane 9: 5 pfu/50 µL of CSF (protocol 4).
Lane 10: 1 pfu/50 µL of CSF (protocol 4).
Lane 11: 50 pfu/50 µL of CSF (protocol 5).
Lane 12: 25 pfu/50 µL of CSF (protocol 5).
Lane 13: 10 pfu/50 µL of CSF (protocol 5).
Lane 14: 5 pfu/50 µL of CSF (protocol 5).
Lane 15: 1 pfu/50 µL of CSF (protocol 5).
Lane 16: -ve control (Vero DNA pretreated by protocol 4).
Lane 17: -ve control (Vero DNA pretreated by protocol 5).
Lane 18: contamination control (S.D.H_2O).
Lanes 1-5, 12-15 were positive

The aim of this study was to investigate several CSF pretreatment techniques to compare the efficiency of recovery of DNA and to assess their advantages and limitations. The ultimate aim of this work was to develop simplified procedure for routine work. Nevertheless there were certain considerations for the protocols as: The techniques provided from 0 to 100% of DNA recovery since efficient DNA revival is crucial when a sample of CSF may contain very low copy of viral DNA. Another limitation upon DNA recovery is time, since certain methods may enable 100% recovery but require long processing time. This has little value for diagnostic laboratories where rapid techniques are applicable to emergency testing are required. Karlinsey *et al*. (1989) for example, have proposed a pretreatment protocol which requires an over night incubation, even though this method gives very high yield of DNA it cannot be applied

Fig. 5: Lane 1: 50 pfu/50 µL of CSF (protocol 6).
Lane 2: 25 pfu/50 µL of CSF (protocol 6).
Lane 3: 10 pfu/50 µL of CSF (protocol 6).
Lane 4: 5 pfu/50 µL of CSF (protocol 6).
Lane 5: 5 pfu/50 µL of CSF (protocol 6).
Lane 6: 50 pfu/50 µL of CSF (protocol 7).
Lane 7: 25 pfu/50 µL of CSF (protocol 7).
Lane 8: 10 pfu/50 µL of CSF (protocol 7).
Lane 9: 5 pfu/50 µL of CSF (protocol 7).
Lane 10: 1 pfu/50 µL of CSF (protocol 7).
Lane 11: -ve control (Vero DNA pretreated by protocol 6).
Lane 12: -ve control (Vero DNA pretreated by protocol 7).
Lane 13: +ve control 50 pfu/10 µL of S.D.H_2O.
Lane 14: +ve control 25 pfu/10 µL of S.D.H_2O.
Lane 15: +ve control 10 pfu/10 µL of S.D.H_2O.
Lane 16: contamination control (S.D.H_2O).
Lanes 1, 2, 4, 5, 7, 9, 13, 14 and 15 were positive

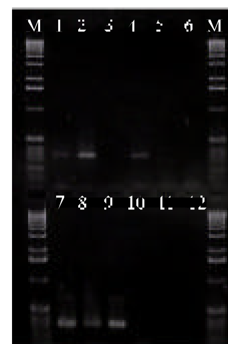

Fig. 6: Lane 1: 50 pfu/50 µL of CSF (protocol 8).
Lane 2: 25 pfu/50 µL of CSF (protocol 8).
Lane 3: 10 pfu/50 µL of CSF (protocol 8).
Lane 4: 5 pfu/50 µL of CSF (protocol 8).
Lane 5: 1 pfu/50 µL of CSF (protocol 8).
Lane 6: -ve control (Vero DNA).
Lane 7: +ve control 50 pfu/10 µL of S.D.H_2O.
Lane 8: +ve control 25 pfu/10 µL of S.D.H_2O.
Lane 9: +ve control 10 pfu/10 µL of S.D.H_2O.
Lane 10: contamination control (S.D.H_2O).
Lanes 1, 2, 4, 7, 8, 9 were positive

in routine laboratories. Efficiency and time are thus complementary and each cannot be considered in isolation.

A significant limitation in pretreatment is ease of processing because complexity can cause mishandling and subsequently false negative or false positive results being obtained. As shown in Fig. 6 protocols proposed by Brice *et al.* (1989) and Rowley *et al.* (1990) are both complex methods therefore above stated risks may occur. Furthermore simplicity is important if they are applied to process large number of samples per day. However, present results showed that simplicity alone is not advantageous because in this project two extremely simple methods of Grimberg *et al.* (1989) *and* Lench *et al.* (1988) did not provide efficient DNA recovery (Fig. 3 and 4).

Proteinase K was used in several different methods for digestion of protein in clinical samples, however the addition of proteinase K itself a protein may actually result in an increase in the final concentration of protein added to PCR cocktail. In this project it was obvious that in those protocols that had suggested addition of 5 μL (20 mg mL^{-1}) of proteinase K, the final protein concentration was higher ($^{O.D260}/_{O.D280}$<1.8) than those (as protocol 1 and 5) without usage of this enzyme. A further limitation with usage of proteinase K is that after addition to the sample incubation at 56°C for 1-2 h is required, thus prolonging processing time. These limitations have led to a diminution in the use of proteinase K, for instance Shibata *et al.* (1988) (protocol 6) suggested incubation of sample at 95°C for 10 min to inactivate protein. Alternatively in another protocol designed by Rowley *et al.* (1990) lysis buffer with a lower amount of proteinase K (1.2 μL) was used for protein digestion in the sample and satisfactory results were obtained (ratio of DNA to protein higher than 1.8) and protein was efficiently removed (data not shown).

In another protocol (No. 2 by Boom *et al.*, 1990) addition of guanidine thiocyanate was proposed for protein digestion followed by silica particle for binding to DNA. The result was reasonable (ratio of 1.47) but the use of guanidine thiocyanate has its own limitations, i.e., in acidic conditions lethal vapor is produced, therefore wastes must be disposed in alkaline solution.

Hence it is suggested that the use of proteinase K for the pretreatment of CSF samples could be avoided. After analysis of samples by PCR in techniques (No. 3 and 4) that utilized this enzyme as the major processing element the results indicated that proteinase K was probably inhibitory for the PCR (Fig. 3 and 4).

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