Egypt. Acad. J. biolog. Sci., 2(1): 1- 8 (2010) **G. Microbiology**
Email: egyptianacademic@yahoo.com **ISSN: 2090-0872**
Received: 20/2/2010 **www.eajbs.eg.net**

Sensitivity of nested PCR toward human cytomegalovirus-DNA in native sera and in extracted DNA of serum samples

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ABSTRACT

PCR has been commonly used for genomic viral diagnosis for its sensitivity and accuracy. It showed a higher sensitivity when compared to virus isolation in tissue culture and also in antigenemia detection. Definitely, DNA samples are critical factor in PCR validity. Out of 84 serum samples subjected to this study and extracted by Wizard® DNA purification mini kit, 27 samples (32.1%) were positive PCR. While, 15 samples (17.9%) only out of the same population study (84) were positive PCR for HCMV DNA in native serum samples (without DNA extraction). This result was confirmed the importance of DNA extraction from serum samples for detection of HCMV which, subsequently lead to more sensitive diagnostic tool of an ongoing HCMV infection.

Keywords: HCMV DNA, HCV RNA, nested PCR, DNA extraction, serum.

INTRODUCTION

Human cytomegalovirus (HCMV) is known to cause mild or asymptomatic infection in most healthy individuals but it can also cause symptomatic disease in congenitally infected or premature neonates as well as in some immunosuppressed hosts (Abdolreza *et al*., 2010).

The most commonly used method to detect HCMV infections was, until recently, conventional cell culture on human fetal lung fibroblasts. The problem with this procedure is that cytopathic effect (CPE) evolves, most of the time, very slowly: it can take up to 21 days to visualize the CPE of HCMV infection in cultured cells (The *et al*., 1995).

Long time ago, HCMV was detected by centrifugation of leucocytes in a shell vial. Immediate early antigens were detected 48 hrs later by using a monoclonal antibody, which recognizes the 72-kDa protein (Gleaves *et al*., 1984). Alternatively, it is possible to detect HCMV antigens, in blood, by using monoclonal antibody to the pp65 HCMV protein (The *et al*., 1995). The conventional technique, using dextran sedimentation, seems to be time consuming: 5 hrs is required to obtain a result. Today, methods based on the direct detection of either the viral genomic DNA or the viral mRNAs synthesized at different times of the viral replication cycle are more and more currently used.

Nucleic acid amplification by PCR methods has become the most widely used diagnostic tool for CMV infection (Caliendo *et al.*, 2000).

The diagnostic value of HCMV DNAemia detected by PCR of leukocytes of immunosuppressed patients has been restricted by its low correlation to HCMV viremia **(**Hamprecht and Gerth, 1994) and HCMV disease (Weber *et al*., 1994). Recent, studies have accepted PCR for detection of HCMV DNA in plasma (Spector *et al*., 1992; Aspin *et al*., 1994) and serum of immunosuppressed patients (Patel *et al*., 1994; Yamamoto *et al*., 2001) as a good tool for this purpose.

During recent years PCR protocols were applied for detection of numerous

human viral and non-viral pathogens (Aquino and Figueiredo1, 2001). The ability to demonstrate the presence of viruses in clinical samples with unprecedented sensitive assays has been the driving force for using PCR as a novel diagnostic procedure. Problems are encountered, however, if PCR is employed for the detection of viruses that establish lifelong latency in the host interrupted by episodes of recurrences (Liliane *et al*., 2001). Thus, during acute infection with human cytomegalovirus (HCMV), viral DNA is readily detected by PCR in peripheral blood mononuclear cells (PBMCs), biopsies, serum, urine and various other specimens (Rogers *et al.*, 1990; Brytting *et al.*, 1992), but positive results may be obtained during asymptomatic reactivations and in latently infected healthy individuals as well (Bevan *et at.*, 1991). Therefore, without further laboratory and clinical data, positive PCR results are difficult to interpret. So, this study was aimed to determine the best clinical samples which give the maximum validity and sensitivity of applied technique (nested PCR).

MATERIALS AND METHODS

Study population:

A total of 84 serum samples were used for monitoring HCMV. The samples were kindly supplied from clinical units of El Demerdash Hospital, Wadii El Neil hospital, and El Moqawelon Hospital, Cairo, Egypt. Fifty three of these samples were positive HCV RNA and 31 were negative HCV RNA. Both positive and negative HCV-serum samples were screened, in this study, for HCMV DNA by nested PCR. DNA extraction**:**

HCMV DNA was extracted from 300 µl serum sample using Wizard® DNA purification mini kit, Promega (Madison, USA), 300 µl serum was added to 900 µl Cell Lysis Solution (included in Wizard® DNA purification mini kit, Promega) and mixed well. After incubation at 30 °C for 10 min, the tubes were centrifuged at 11500 xg for 20 Sec at room temperature; the supernatant was removed without distributing the visible white pellet.

300 µl Nucleic lyses solution (included in Wizard® DNA purification mini kit, Promega) was added to the tube containing the white pellet, mix well, then incubated at 37 °C. 1.5µl of RNase solution (included in the same kit) was added and the sample was mixed by inverting the tube 2-5 times. The mixture was incubated at 37 °C for 15 min, and then cooled at room temperature. 100 µl protein precipitation solution (included in the kit) was added and vortex for 10-20 min. After centrifugation at 11500 xg for 3 min at room temperature, the supernatant was transferred to a clean 1.5ml microcentrifuge tube containing 300µl isopropanol, the tube was mixed well, then centrifuged at 11500 xg for 1 min. The DNA was visualized as a small white pellet. The supernatant was removed and one sample volume of 70% ethanol was added to the pellet, then the mixture was centrifuged at 11500 xg, and then the ethanol supernatant was removed. 100 µl DNA dehydrating solution (included in kit components) was added and DNA was rehydrated by incubating at 65 °C for 1h, DNA was left over night at room temp, and then stored at -20 °C until use.

Nested PCR:

Nested PCR for serum samples to detect HCMV DNA was carried out according to methods of Nelson *et al*., (1997) and Jones *et al*., (2000). The reaction mixture of the qualitative PCR contained, in total volume of 25 µl, 5 µl 10X buffer (75mM Tris-HCl pH 8.0, 50mM KCl, 15mM $MgCl_2$), 0.5 µl 50mM dNTP mix, 0.25 µl of primers gB1: 5 µl GAGGACAACGAA ATCCTGTTGGGCA 3µl and gB2: 5`GTCGACGGTGGAGATACTGC TGAGG 3µl (Bioneer, Atlantic Avenue, Alameda, USA), 5 µl DNA solution

(DNA template), 14.15 µl distilled water, and 0.1 µl 2U of Taq DNA polymerase (Bioneer, USA) were added.

The thermal cycling protocol was applied as follows: pre-denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 sec, annealing at 57 °C for 30 sec, Extension at 72 °C for 60 sec and final extension at 72 °C for 4 min. Two microliters of the 1st PCR product were used in a nested-PCR containing the same components as mentioned above, except for internal primers gBn1: 5 µl ACCACCGCACTGAGGAATGTCAG 3µl and gBn2: 5µl TCAATCATGCGTTTG AAGAGGTA 3 µl (Bioneer, Atlantic Avenue, Alameda, USA), at the same thermal cycling protocol.

PCR Amplification of serum samples without DNA extraction:

1ml of human serum sample was boiled at 95°C for 2 min, centrifuged at 11500 xg for 5 min. 5 µl of the supernatant was used as DNA template in the same nested PCR protocol that mentioned above.

RESULTS

Detection of HCMV DNA by nested PCR:

a. In DNA extracted from serum sample:

Results in Fig. 1 showed that 27 out of 84 tested samples gave positive HCMV-DNA using nested-PCR technique for DNA extracted from serum samples. Among those positive cases, 20 of them (74.1%) were positive HCV-RNA. The remaining 7 cases (25.9%) were negative HCV-RNA (Table 1).

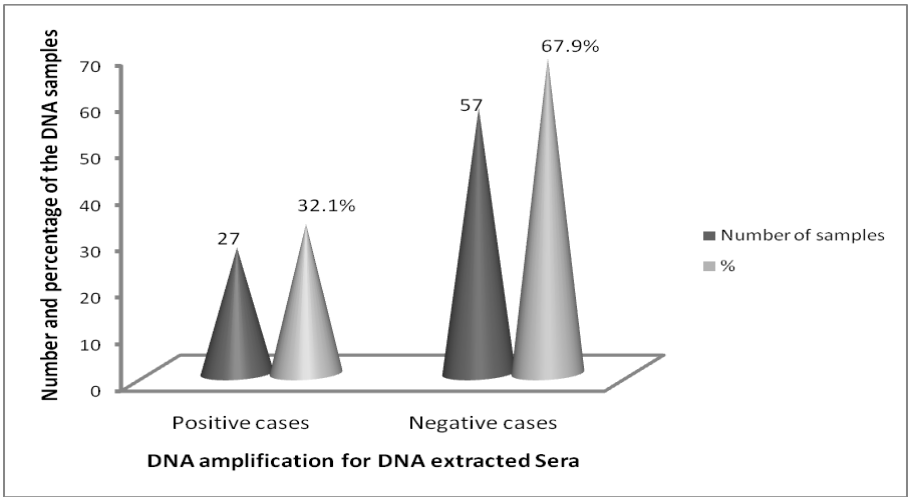

Fig. 1: Detection of HCMV-DNA from extracted DNA of serum samples using nested-PCR

Table 1: Detection of co-infection of HCMV and HCV in positive cases of HCMV infection.

No. of positive HCMV-DNA	Positive DNA amplification for DNA extracted sera	
	Positive HCV RNA	Negative HCV RNA
27	20 (74.1%)	7 (25.9%)

a. In native serum samples:

Results in Fig.2 showed that the native serum samples (without extraction of HCMV-DNA) gave considerable reduction in the number of positive HCMV infection comparing with the previous cases (see Fig. 1). Where 15 out of 84 tested samples (17.8%) were positive for HCMV-DNA. The majority of cases were negative for HCMV-DNA (Table 2).

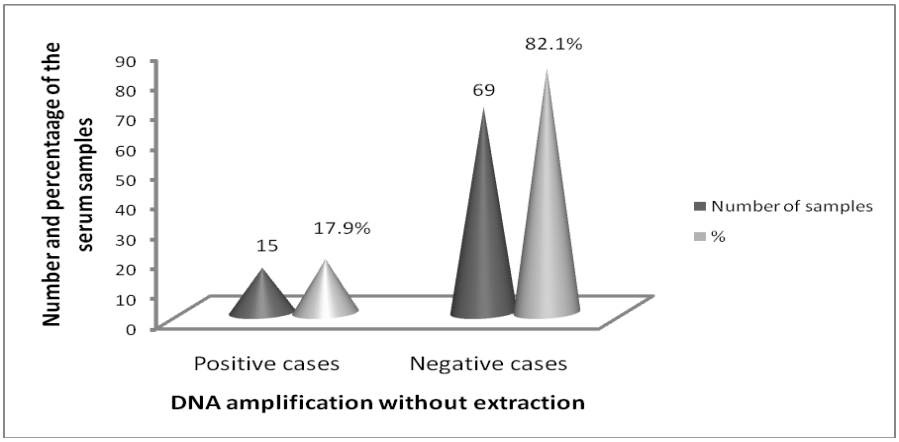

Fig. 2: Detection of HCMV-DNA in native serum samples using nested-PCR.

Table 2 : Detection of co-infection of HCMV and HCV in Positive cases of HCMV infection.

No. of positive HCMV-DNA	Positive DNA amplification in native serum samples	
	Positive HCV RNA	Negative HCV RNA
15	12 (80%)	3 (20%)

Nested-PCR indicated that all tested positive HCMV-DNA samples were also positive (100%) when DNA was extracted from serum samples. Whereas only 15/27 (55.6%) were positive when native serum samples were used without DNA extraction (Table 3). These results confirmed the necessity of extraction of viral genome (DNA) before amplification by nested-PCR. Results in Fig.3 showed that the positive samples gave PCR-product at 100bp as an expected size of HCMV-DNA fragment.

Table 3: Detection of HCMV DNA in some selected samples showing DNA amplification using either DNA extraction kit or native unprocessed sera.

Nested PCR	None (native Sera)	extraction (Wizard kit)
Positive	15 (55.6%)	27 (100%)
Negative	12 (44.4%)	0 (- %)

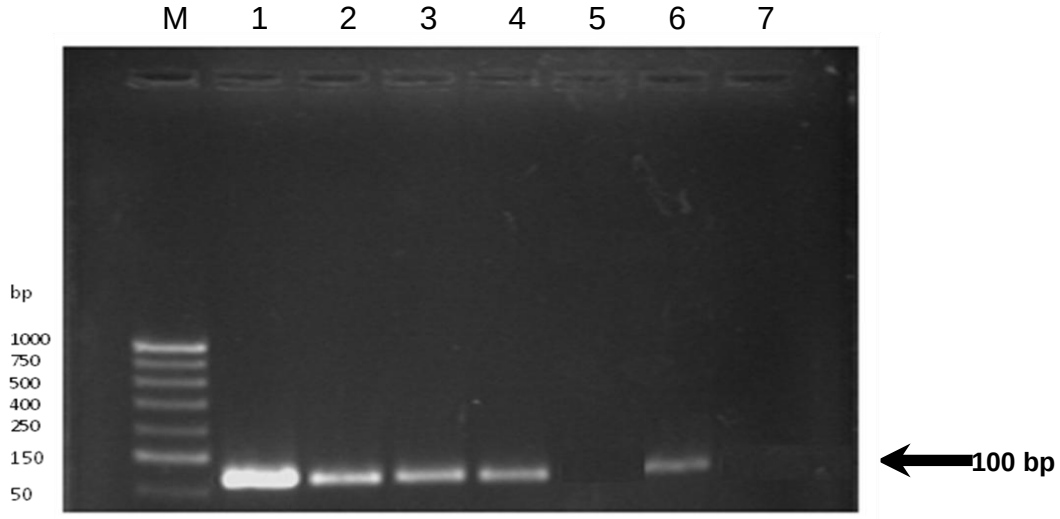

Fig. 3: Nested PCR results of HCMV DNA in serum samples Lanes 1, 2, 3, 4, 6 were positive for HCMV DNA, Lanes 5, 7 were negtive for HCMV DNA. M= DNA marker

DISCUSSION

HCMV is detected earlier by PCR than by other techniques (Gerna *et al*., 1991). The uniplex PCR for HCMV DNA detection followed by confirmatory nested-PCR with gB primers has been used for processing urine, serum, plasma, saliva, milk and other secretion samples, showing high sensitivity and specificity. These sensitivity and specificity rates are higher than those reported for HCMV serological tests (Yamamoto *et al*., 2001).

There is an urgent need for technical standardization of procedures used for isolation and amplification of DNA. It seemed that the influence of methodological aspects such as DNA extraction procedures are important for the sensitivity and specificity of HCMV DNA detection from cell free sera by PCR (Klaus *et al*., 1997).

Several techniques, using different clinical samples have been employed to detect HCMV-DNA either by boiling serum samples without DNA extraction (Spector *et al*., 1992, Aspin *et al.,* 199 1994, Freyuth*et al.,* 1994) or by using recently DNA commercial kits (Bayram *et al.,* 2009). Two decade ago, Ishigaki*et al.,* (1991) demonstrated that the detection of HCMV-DNA in serum or plasma samples without any performance extraction of DNA has a disadvantage regarding the lower sensitivity of applied technique.

In this study, the percentage of positive nested-PCR of HCMV DNA extracted from serum samples were higher than that of positive cases for HCMV DNA without DNA extraction, indicating that the sensitivity of HCMV DNA amplification from DNA extracted serum was higher than that of amplification from native sera samples. The false negative nested-PCR (44.4%) has been shown with the native serum sample. Whereas, no negative results was shown with the extracted HCMV-DNA with Wizard® DNA purification mini kit. It is possible that serum components acted as non-specific, non-enzymatic PCR inhibitors which were not eliminated by the thermal inactivation, but were eliminated after Wizard extraction (Evans *et al*., 1999).

It was reported that, the hemolytic serum of HCV patients contains a number of PCR inhibitors, one of which is hemin. Hemin, the prosthetic group of hemoglobin, reversibly binds to *Taq* polymerase and is a potent inhibitor of the enzyme (Byrnes *et al*., 1975). Such hemin is released from erythrocytes following hemolysis and is frequently associated with the withdrawing of blood. In addition, hemin inhibits viral reverse transcriptase activity in HIV-infected patients (Levere *et al*., 1991 and Klein *et al*., 1997).

Since the sensitivity of PCR for the amplification of HCMV DNA sequences in serum may often be reduced due to the presence of inhibitory factors. Still the method of DNA extraction has perfect effect to detect the target nucleic acid in clinical diagnostic samples (Klein *et al*., 1997).

Interestingly, the number of samples with positive HCMV-DNA amplification in cases of DNA extracted sera of HCV patients was higher than that from native sera of the same patients. This may be attributed to the effect of Wizard® DNA purification mini kit in decreasing the effect of inhibitors on detection of HCMV DNA in sera of HCV patients. Similar results were reported by Gerna *et al*., (1994), Zipeto *et al*., (1995), Klaus *et al*., (1997) and Klein *et al*., (1997).

CONCLUSION

This study concluded that, detection of HCMV DNA by nested PCR from native sera without DNA extraction was less sensitive than that by using DNA extraction kit. DNA extraction and

purification methods had significant influence on the quality of DNA. DNA purification kits improved the accuracy of nested PCR results in clinical samples.

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ARABIC SUMMARY

حساسية تفاعل البلمرة المتسلسل في تحديد الأصابة بفيروس السيتوميجالوا في العينات المستخلص منها الجينوم الفيروسي

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يستخدم تفاعل البلمرة المتسلسل (PCR) عادة في تشخيص الأمراض الفيروسية نظراً لحساسيته و دقته. و تعتبر تقنية التفاعل المتسلسل أفضل التقنيات للحصول علي نتائج مؤكدة نظراً لكونه يتعامل مع الجينوم الفيروسي بشكل مباشر خلال خطوات التقنية المتعارف عليها. و بالنسبة للتشخيص الدقيق لفيروس السيتوميجالو وجد أن تقنية PCR تعطى نتائج دقيقة بالقياس لإستخدام التقنيات التشخيصية الأخري التقليدية كالكشف عن الفيروس في خلايا مزارع الأنسجة أو في بلازما أو أمصال الدم دون إستخلاص الحامض النووي الفيروسي . وقد تأكد هذا المعني معمليا بأستخدام تفنية nested-PCR حيث أن الكشف عن الحامض النووي لفيروس السيتوميجالو أعطى نتائج إيجابية في 27 عينة بالنسبة للعدد الكلي وهو 84 عينة بنسبة 32.1%. بينما كان عدد العينات الموجبة للتفاعل بدون إستخلاص الحامض النووي هو 15 عينة بنسبة 17.9%. وتأكد هذه الدراسة علي أهمية إستخلاص الحامض النووي الفيروسي من عينات البلازما أو المصل للكشف الدقيق عن إيجابية وجود الأصابة من عدمه دون الرجوع إلي الطرق التقليدية التي تستهلك كثيراً من الوقت مع بعض النتائج المضللة.