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RESEARCH ARTICLE

Molecular Study of CMV Isolated From Women with Repeated Miscarriage in Relation to Immune Response Molecule TLR2

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Abstract

This study was constructed to discuss a Molecular Study of Cytomegalovirus isolated from women with repeated miscarriage in relation to immune response molecule Tall like Receptor2. About (100) blood samples from women suffering from infection with Cytomegalovirus were collected from infertility clinic of Kamal Al –Sammaraee hospital and (50) samples from normal subjects served as control for comparison. Test subjects were divided into two age groups: 20-30 years old and 31-40 years old. The women distributed as (60) samples of infertile and (40) samples as miscarriage women, included molecular analysis by using polymerase chain reaction technique (PCR) for amplification two viral genes glycoprotein B (gB) that showed molecular weight 72bp and immediate early gene (IE2) that showed molecular weight 92bp. The amplification for TLR2 primer region showed molecular weight 419bp. After successful amplification of TLR2 gene good quality products were selected to be sequenced. The result showed that there is no mutation at TLR2 only replacement of C instead of G in wild type. PCR amplified for ILT2 primer region showed molecular weight of 285bp. Among ten Iraqi patients, only six give positive result when compared with healthy control; the type of mutation is deletion and substitution. The percentage of mutation types were deletion mutation (13.3%) and (86.7%) for substitution mutation. the effect of mutation was missense mutation (72.7%), Silent mutations (15.1%) and deletion mutations (12.2%).

Introduction

Human cytomegalovirus (HCMV) is a ubiquitous member of the Herpesviridae family subfamily BetaherpesVirinae. HCMV is the major infectious cause of congenital infection and hearing loss in children as well as an important pathogen in immuncompromised patients (Revello and Gerna, 2004). The viral nucleocapsid containing a linear double stranded DNA of 236 kb and is surrounded by a proteinaceous tegument which is itself enclosed by a loosely applied lipid bilayer (Davison *et al*., 2003). HCMV has an extremely broad tissue tropism that allows it to infect nearly every organ system in the body (Sinzger *et al*., 2000). It is a major cause of postoperative disease in chemically immunosuppressed transplant recipients and greatly increases the risk of graft rejection (Razonable and Paya, 2003). HCMV is also a leading cause of congenital birth defect and infection during the first trimester of pregnancy often results in neurological and cognitive disorders in the developing child (Streblow *et al*., 2001).HCMV can be transmitted by close personal contact and by blood transfusion or organ transplantation. HCMV is frequently transmitted from mother to child either in utero or during the perinatal phase. Perinatal transmission often results in severe disturbance of development and disease that may become manifest at birth such as

thrombocytopenia, hepatitis, splenomegaly and microcephaly (Stagno *et al*., 1985). Tall like Receptors (TLRs) are the activators of signal transduction pathways that lead to the expression of antimicrobial/antiviral genes and the induction of inflammatory cytokines (Akira, 2001). TLRs detect microorganisms on the basis of unique molecular structure termed pathogen associated molecular pattern (PAMPs) (Schwandner *et al*., 1999), viral genomic nucleic acids are the one major class of PAMP. TLR3 (dsRNA), TLR7 (ssRNA), TLR8 (ssRNA) and TLR9 (CpG DNA) signal from the endosome where degradation of virus particles exposes the viral genome for detection by this panel of TLRs (Diebold *et al*., 2004). A miscarriage is the spontaneous loss of a pregnancy between conception and 24 weeks into pregnancy. CMV is associated significantly with more than 70% of abortion cases (Goodrich *et al*., 2004). Furthermore 60% of pregnant women reported during first gestation trimester and begin to decline significantly in the late gestation for virus (Pass *et al*., 2006).

Materials and Methods:

- **Patients Selection and Blood Sample Collection.**

Blood Samples were collected from one hundred (100) women suffering from CMV infection and miscarriage, 50 healthy women as a control (healthy), their ages ranged between (20-40) years. Samples were subjected to centrifugation at 2000 rpm for 10min.The serum was separated and stored at -20^{0}C. All Samples were subjected for HCMV antibodies using ELISA techniques. In case of blood with EDTA, it was stored at -20^{0}C until used for DNA extraction. The Samples were obtained from infertility clinic of Kamal Al-Sammaraee hospital. The collection period extended from November 2013 to March 2014.

- **DNA Extraction**

Total cellular DNA was extracted from blood samples by using the Reliaprep Blood genomic DNA MiniPrep System from Promega USA, estimation the concatenation and purity of the extracted DNA were measured by using nanodrop (UVIS Drop\ACTGene\USA).

- **PCR Amplification**
- **Primers**

sequences of the primers used to amplify portion of Cytomegalovirus gene:

DNA gel electrophoresis (Maniatis *et al*., 1982).

Statistical Analysis:The Statistical package for the social sciences (ANOVA version15).

Result and discussion

Name	Oligonucleotides	Tm^{0}C	GC %	Sequence (5'-3')
gB	Forward primer	57.9	45.8	AGGTCTTCAAGGAACTCAGCAAGA
gB	Reverse primer	54.3	42.8	CGGCAATCGGTTTGTTGTAAA
IE2	Forward primer	56.9	55.00	GAGCCCGACTTTACCATCCA
IE2	Reverse primer	57.8	68.7	CAGCCGGCGGTATCGA
TLR2	Forward primer	57.6	54.00	CTGTGCTCTGTTCCTGCTGATC

TLR2	Reverse primer	55.2	53.00	TGAGAATGGCAGCATCATTG TT
ILT2	Forward primer	53.8	55.00	TGAGAGTGGTGGGAATGCAC
ILT2	Reverse primer	51.7	50.00	AACCCAACGCAATTTCCAGC

Molecular detection of Cytomegalovirus by PCR technique

Cytomegalovirus (CMV) is associated significantly with more than 70% of miscarriage cases. If the mother is healthy she may not show symptoms herself but in the womb the virus may affect the fetus's developing brain, nervous system and this is the major cause of congenital infection and hearing loss in children . PCR is the most widely used technique for detection DNA of CMV; the PCR results were interpreted by the presence or absence of specific bands of amplified gene on 2% agarose (Sam book, 2012). Several comparative studies have demonstrated that glycoprotein B (gB) primer has high diagnostic sensitivity for CMV infection (Distefano *et al*., 2004) and has a largely conserved nucleotide sequence (Coyle *et al*., 2002). PCR used targets the conserved region that lies upstream of antigenic domain1of the gB gene between base pairs 1541and 1612 (Britt *et al*., 2005). PCR amplified showed molecular weight of 72bp as shown in figure (1):

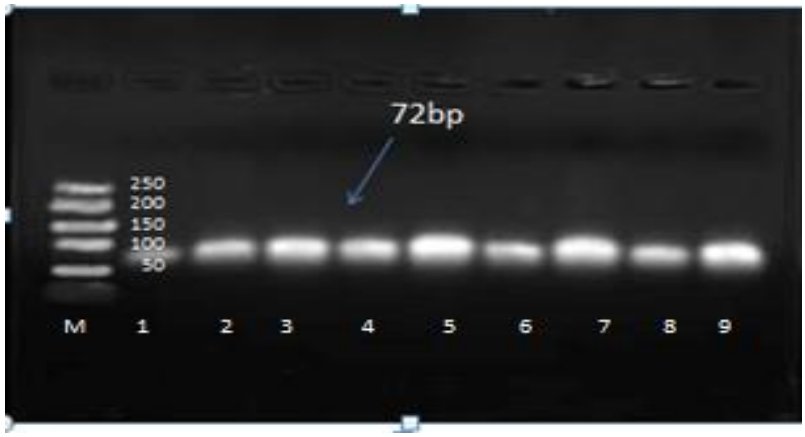


Figure (1): PCR amplified for gB primer, PCR detection of virus DNA in ethidium bromide stained (2% agarose gel, 10 minutes at 100 V and then lowered to 70 V, 50 minutes). Line M (50bp) marker, 1-9 for miscarriage women.

Another primer used in this study is the immediate early 2(IE2) primer were specific for exon 5 of the IE2 gene of CMV, several comparative studies have demonstrated that this pair of primers has been evaluated for its specificity and sensitivity against CMV clinical isolates and other herpesviruses (Boppana *et al*., 2010). PCR amplified for IE-2 primer region showed molecular weight of 92bp as shown in figure (2):

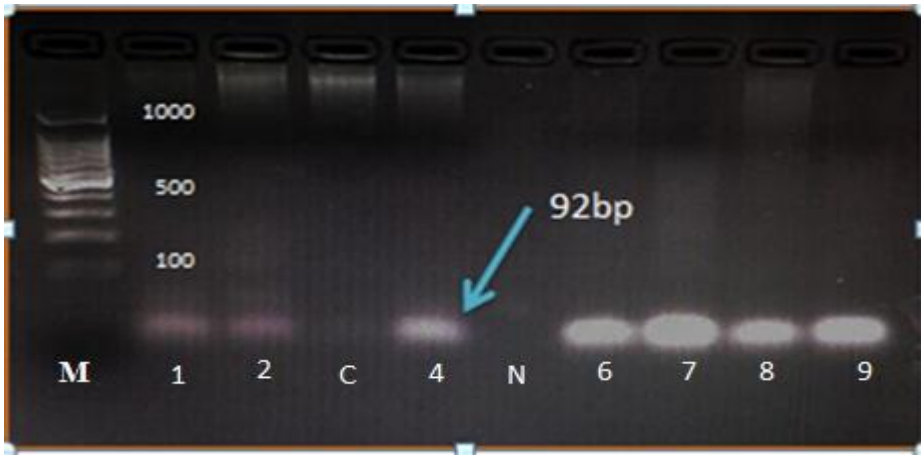


Figure (2): PCR amplified for IE-2 primer, (2% agarose gel, 10 minutes at 100 V and hen lowered to 70 V, 50 minutes). PCR products visualized under U.V light after staining with ethidium bromide. M: 100 bp DNA marker; lane1,2,4,6,7,8,9: for miscarriage women, lanes C for healthy (control) and lane N for negative control .

Sequencing of TLR2 genes

The major pattern recognition receptors (PRR) family is represented by Toll – like receptor which involves in innate immunity of the individual. Sometimes such immune response against microorganisms may have significant impact on pregnancy success especially infections affecting intrauterine that may lead to certain disorders.

Recent experimental observations indicate that TLRs participate in innate immunity against CMV infection and in the control of CMV infection through the release of antiviral cytokines and the regulation of adaptive immunity. The presence of functional polymorphisms in TLR2 would impede this immune response and clinically lead to higher levels of CMV replication and symptomatic CMV disease . The human TLR2 gene was located in chromosome 4 (Texereau *et al*., 2005). PCR amplified for TLR2- primer region showed molecular weight of 419bp as shown in figure (3):

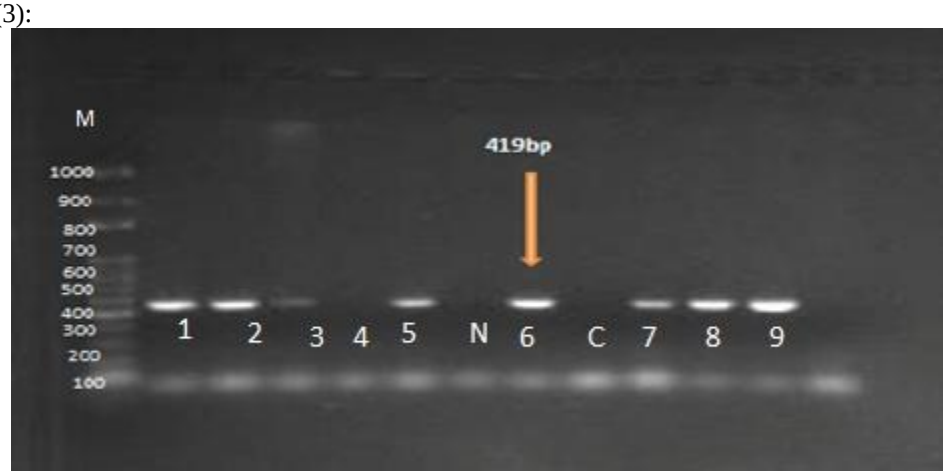


Figure (3): PCR amplified for TLR-2 primer, (2% agarose gel, 10 minutes at 100 V and hen lowered to 70 V, 50 minutes). PCR products visualized under U.V light after staining with ethidium bromide. M: 100 bp DNA marker; lane1,2,3,4,5,6,7,8,9: for miscarriage women, lanes C for healthy (control) and lane N for negative control .

After successful amplification of TLR2 gene good quality products were selected to be sequenced. Only 3 out of 10 samples were failed to be sequenced properly. Sequencing was carried out at NICEM (National Instrumentation Center for Environmental Management) using their ABI3730XL instrument (Applied Biosystems, USA). Results were directly compared with Iraqi healthy control by using the program Mega 6.there is result showed that no mutation at TLR2 only replacement of C instead of G in wild type as shown in figure (4).

ure (4) compared between control and patient samples.

This result agree with Pabst *et al*., (2009) who pointed that the TLR2 polymorphisms in chronic obstructive disease have no obvious effect on individual susceptibility to disease especially those Caucasian groups. Previous studies showed this SNP to be absent in Asian population such as Taiwan (Cheng *et al*., 2007).

Molecular diagnosis for ILT2 Receptor

The UL18 gene of human cytomegalovirus encodes a protein similar to major histocompatibility complex (MHC) class I genes. This associated with 2 – microglobulin which it's stability depends upon peptide loading (Browne *et al*., 1990). The receptor for UL18 is ILT2, UL18 protein binds to immunoglobulin-like transcript 2 (ILT2) an inhibitory receptor present on B cells, monocytes, dendritic cells, T cells and NK cells that also recognizes classical and non-classical MHC molecules (Colonna *et al*., 1997). ILT2 is a 110-kDa transmembrane protein with four immunoglobulin domains in the extracellular portion and four ITIMs in the cytoplasmic tail that allow it to function as an inhibitory molecule. UL18 may play a role in viral immune evasion, but its real function is unclear. PCR amplified for ILT2 primer region showed molecular weight of 285bp as shown in figure (5):

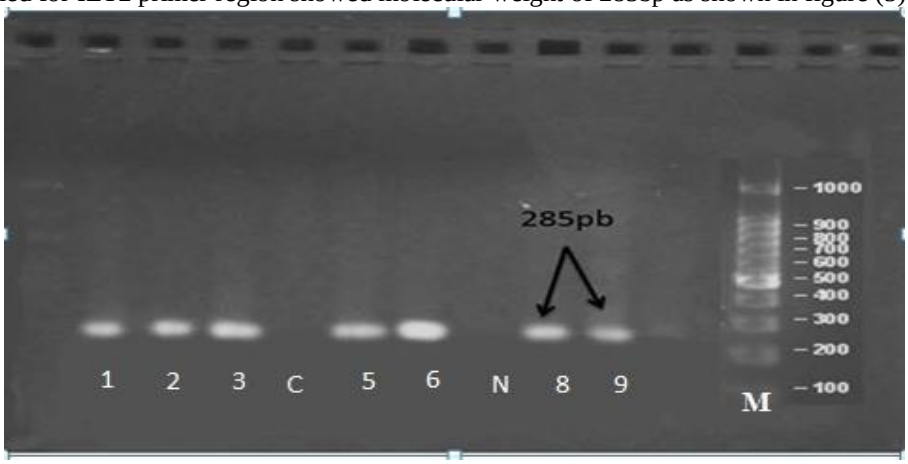
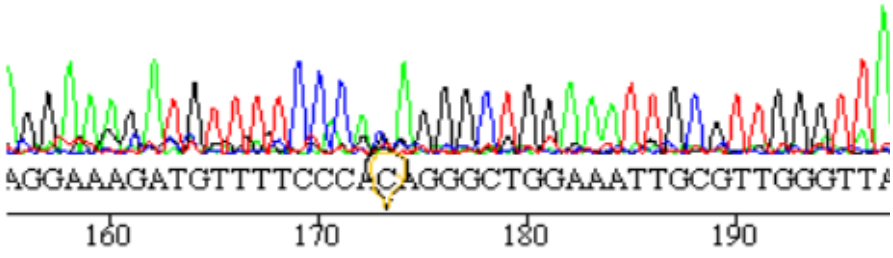


Figure (5): PCR amplified for ILT2 primer with ethidium bromide stained (2% agarose gel, 10 minutes at 100 V and then lowered to 70 V, 50 minutes). Line M (100bp) marker, 1,2,3,5,6,8,9 for patient, 4 for healthy (control) and lane 7 for negative control.

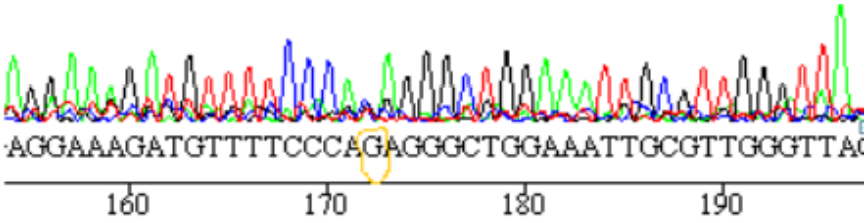
Type of mutation

The mutations in CMV Patients were undetectable during diagnosis by PCR. The PCR products of ILT2 were screened by sequencing CMV patients. Sequencing was carried out at NICEM (National Instrumentation Center for Environmental Management) using their ABI3730XL instrument (Applied Biosystems, USA). Results were directly compared with Iraqi healthy control by using the program Mega 6.



Control

AGG\ AAA \GAT \GTT \TTC\ CCA\ CAG\ GGC\ TGG\AA A\ TTG\ CGT\TG G



AGG\ AAA \GAT\ GTT\ TTC\ CCA\ GAG\ GGC\ TGG\ AAA\ TTG\ CGT\ TGG **Figure (6) compared between control and patient sample.**

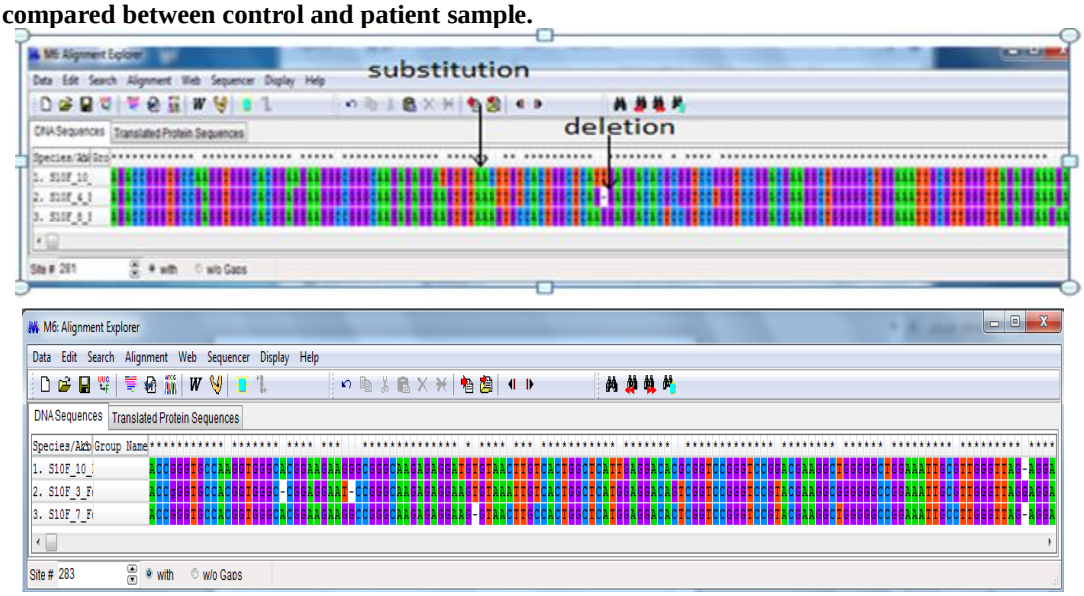


Figure (7) point mutation as illustrated by the program Mega 6.

Table (1): point mutations detect in patient 7 and 3.

No. of patients samples	Wild type	Mutant type	Change in amino acids	site Of N.A.	Type of Mutation	Effect on translation
7	AGG	CGG	Arg- Arg	12	Substitution	Silent
	GAT	GAA	Asp-Glu	46	Substitution	missense
	GTG	G - G	Val-Deletion	48	Deletion	Frame shift
	GTC	GCC	Val-Ala	57	Substitution	missense
	TTG	TGG	Leu- Trp	69	Substitution	missense
	CGC	GTC	Arg-Val	77-78	Substitution	missense
	GAC	TAC	Asp- Tyr	92	Substitution	missense
3	AAG	ACG	Lys-Thr	12	Substitution	missense
	GCA	GC -	Ala- deletion	20	Deletion	Frame shift
	AAG	AGG	Lys- Arg	25	Substitution	missense
	AAG	AAT	Lys- Asn	29	Substitution	missense
	GGC	- CC	Gly-deletion	30-31	Deletion-Substitution	Frame shift
	GAT	GAA	Asp- Glu	46	Substitution	missense
	CTT	ATT	Leu-lle	53	Substitution	missense
	TTG	TGG	Leu-Trp	69	Substitution	missense
	CGC	GTC	Arg-Val	77-78	Substitution	missense
	GAC	TAC	Asp-Tyr	92	Substitution	missense
	TGG	GGG	Trp-Gly	101	Substitution	missense

Table (2): point mutations detect in patient 4 and 8.

No. of	Wild	Mutant	Change in amino	site Of	Type of	Effect on

patients samples	type	type	acids	Nucleic acid	Mutation	translation
4	CAA	CGA	Gln- Arg	13	Substitution	missense
	AGA	AGG	Arg- Arg	29	Substitution	Silent
	TGT	AGT	Cys-Ser	48	Substitution	missense
	ACT	AAT	Thr-Asn	55	Substitution	missense
	ATT	AG -	Lle- deletion	71	Deletion	Frame shift
	GGG	TGG	Gly-Trp	87	Substitution	missense
8	CAA	CGA	Gln-Arg	13	Substitution	missense
	AGA	AGG	Arg-Arg	29	Substitution	Silent
	GCG	CCG	Ala-Pro	33	Substitution	missense
	TGT	AGT	Cys-Ser	48	Substitution	missense
	ACT	AAG	Thr-lys	55-56	Substitution	missense
	TGT	TGC	Cys-Cys	59	Substitution	Silent
	ATT	AAG	Lle-lys	70-71	Substitution	missense
	ACG	ACT	Thr-Thr	80	Substitution	Silent
	CGG	CCG	Arg-Pro	82	Substitution	Missense

The current study utilized forwared primer for sequencing, six samples out of 10 were compared with iraqi healthy control at the first (100) bp regions. Among them, four patients displayed point mutations of deletion and substitution as shown in figure (3-10) while others were normal since no mutations were detected. This result agrees with Mar Vale´s- *et al*. (2005) reported that the UL18 gene varies significantly, amino acid substitutions were found between virus isolates. Cristina *et al*. (2006) also reported that UL18 derived from HCMV strain show amino acids difference and point mutation.

Percentage of mutation

Analysis of the result obtained from sequencing four Iraqi patients exhibited the existence of much genetic alteration. Two types of mutations namely deletion and substitution were present. Table (3-5) shows the percentage of mutation types that showed 86.67% for substitution mutation and13.33% for deletion mutation.

Table (3): percentage of mutation type.

Type of Mutation	Percentage%
Substitution	86.67
Deletion	13.33

Effect of mutation:

The effect of mutation indicates that there was a missense mutation that causing impact on phenotype leads to replacement in amino acids, this effect appears in current study with high level 72.7% as shown in Table (3-6) Silent mutations15.1% result when the codon in wild type coded for the same amino acids in mutant type. The deletion mutations lead to frame shift which represented 12.2%.

Table (4): percentage of Effect of mutation.

Effect of Mutation	Percentage %
missense	72.7
Silent	15.1
Frame shift	12.2

Conclusions

Considering PCR test as the cutting edge in diagnosis patient with CMV infection and Point mutations were detected including deletion and substitution causing missense, silent and frame shift, so making CMV check on women and men who about to get married mandatory.

References

- Brown HL, and Abernathy M P Cytomegalovirus infection. NCBI, Pub. Med. ,(1998); 22(4): 260-266.
- Boppana S B, Foler K B, Pass R F, Rivera L B, Bradford R D,Lakeman F D and Britt W J. Congenital cytomegalovirus infection: the association between virus burden in infancy and hearing loss. NCBI, Pub. Med. (2005); 146(6):817-823.
- Cheng P, Eng H, Chou M, You H,and Lin T.Genetic polymorphisms of viral infection associated Toll like receptors in Chinese population. Transl. Res. (2007); 150:311-318.
- Coyle P, Wyatt D, Ong G, Maxwell A, Mc McCaughey C, and O'Neill H.A nested primer set targeting the cytomegalovirus glycoprotein B gene. J.Clin Virol. .(2002); 25(1):95-96.
- Cooper T G, Noonan E, von Eckardstein S, Auger J, Baker H W, Behre H M,. World Health Organization reference values for human semen characteristics. NCBI, Pub. Med, (2010); 16 (3): 231–245.
- Cristina C, Adnane A, Anette W, Mehrdad M, Tatyana S, Mei-Ling H, David C, Klas K and Ennio C. Spontaneous mutations in the human CMV HLA class I homologue UL18 affect it's binding to the inhibitory receptor LIR-1/ILT2/CD85j. Eur. J. Immunol. (2006); 36: 732–74.
- Davison A J, Dolan A, Akter P, Addison C, Dargan D J, Alcendor D J, McGeoch D J and Hayward G S. The human cytomegalovirus genome revisited: comparison with the chimpanzee cytomegalovirus genome. NCBI, Pub. Med. (2003); 84:17–28.
- Distefano A, Alonso A, Martin F, Pardon f. Human cytomegalovirus: detection of congenital and perinatal infection in Argentina. NCBI, Pub. Med. (2004); 4:11 .
- Munro S, Hall B, and Whybin L. Diagnosis of and screening for cytomegalovirus infection in pregnant women. J. Clin. Microbiol. (2005); 43(9):4713-4718.
- Pabst S, Yenice V, Lennarz M, Tuleta I, Nickenig G, Gillissen A. Tall like receptor 2 gene polymorphisms Arg677 Trp and Arg753 Gln in chronic obstructive pulmonary disease. Lung (2009); 187:173-178.
- Prentice H, and Kho P. Clinical strategies for the management of cytomegalovirus infection and disease in allogeneic bone marrow transplant. NCBI, Pub. Med. (1997);19:135-42.
- Razonable R R, and Paya C V. Herpesvirus infections in transplant recipients: current challenges in the clinical management of cytomegalovirus and Epstein Barr virus infections. NCBI, Pub. Med. (2003);10: 60–65.
- Sinzger C, Kahl M, Laib K, Klingel K, Rieger P, Plachter B and Jahn G. Tropism of human cytomegalovirus for endothelial cells is determined by a post entry step dependent on efficient translocation to the nucleus. J. Gen. Virol. (2000); 81: 3021–3035.
- Stango S, and Whitley R J. Herpesvirus infections of pregnancy. Part I: Cytomegalovirus and Epstein-Barr virus infections. NCBI, Pub. Med (1985).
- streblow D N, Orloff S L, and Nelson J A. Do pathogens accelerate atherosclerosis? J. Nutr. (2001); 131(10): 2798–2804.
- Voler A, and Bidwell J. Manuel of Clinical Immunology. (1985); Chapter 69. Am. Soc. Microbiol. :506.