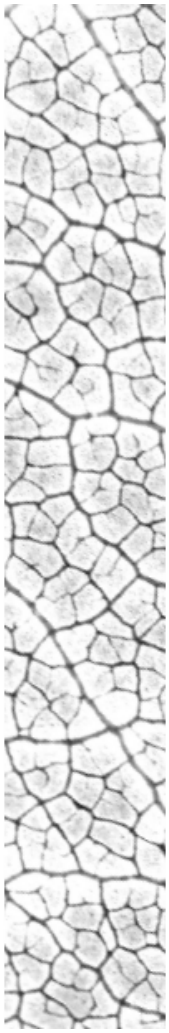

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Determination of congenital cytomegalovirus infection and virus genotypes in newborn urine samples

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Abstract
Congenital cytomegalovirus (cCMV) infection may cause significant clinical consequences in newborns, not only at birth, but also later as neurological sequelae. It is very important to establish a strategy for rapid and accurate screening of congenital CMV infections for determining the risk factors that may threaten the newborns life. To our knowledge most of the previous studies of a CMV infection in Iraq have been based on serological and immunological tests. Our current study is one of the few molecular studies focused on the molecular diagnosis of the virus and determination of the dominant viral genotype in Baghdad province. n the recent study active CMV infection was detected in 24 samples (12%) in real time PCR with significant Ct value <35. Amplicons sequencing was indicated about twelve out of 20 (60%) recipients had CMVGT2 (gB2) genotype; 6 out of 20 (30%), recipients had CMVGT4 (gB4); 2 out of 20(10%), CMVGT1 (gB1). Our finding indicated that the most frequent genotypes were gB2 followed by GB4. Real time PCR assay was very accurate, rapid, and sensitive method of CMV early diagnosis, also M-nPCR and sequencing was a specific assay for CMVGT genotyping.

Keywords: congenital cytomegalovirus infection, human cytomegalovirus, sensor neural hearing loss, neonatal screening

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INTRODUCTION

Human cytomegalovirus (CMV) is the most common etiological agent for viral intrauterine, and congenital infections recently in the world (Allice et al. 2008, Zuylen et al. 2014). CMV infection occurs in 0.2 to 2% of newborns in many developed countries, the a symptomatic prevalence represented about 0.64% of all live births and causes obvious clinical consequences not at birth only but also later as neurological sequelae, including sensor neural hearing loss (SNHL) and developmental delay (Pass and Arav-Boger 2018, Rawlinson et al. 2016). Up to half of symptomatic infants are suffering of some degree of psychomotor, cognitive disabilities with visual impairment (Bate et al. 2010). Clinical management is dependent mainly on the detection of CMV in the amniotic fluid and the fetal infection acquired in late stage of pregnancy may be asymptomatic in newborns, whereas the infection acquired in the first half term of pregnancy may carry a higher risk of symptomatic fetuses (Gindes et al. 2008). The clinical manifestations of congenital CMV at birth are jaundice, microcephaly, petechial rash, hepatosplenomegaly, and infants born small size for gestational age. Also 13% of newborns with this infection showed symptoms at birth, and the rest of them 87% were asymptomatic or in many cases had subclinical manifestations of this infection; unfortunately, many of these cases will go undiagnosed in the absence of routine neonatal clinical screening programs. However, 6–23% of these asymptomatic new born cases will later develop some degree of SNHL (Fowler and Boppana 2006), and can show possibly progressive deterioration over time. Early diagnosis of the infection contributes to its treatment within the first 4 weeks of life, can reduce or prevent progression of SNHL and may improve long-term neurodevelopmental outcomes (Kimberlin et al. 2015).

MATERIALS AND METHODS

Urine Samples collection: Two hundred urine samples for both genders were collected from Al-Alawi Maternity Hospital and Karkh Maternity Hospital during the period between mid of May to the mid of September 2018. For cCMV infection, PCR detection virus isolation technique was used within 1-3 weeks of neonatal age. The urine was collected in a plastic bag with a sticky strip on one end, made to fit over the newborn genital area, and then the urine was emptied in a sterile disposable plastic container and preserved at 4°C.

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Table 1. The Primers used in CMV genotyping Nested PCR

Primers	Oligonucleotide	Amplicons size bp	Nested Round
Reverse CMV Q1F	5' TTT GGA GAA AAC GCC GAC3'	751	First round
Reverse CMV Q1R	5'CGC GCG GCA ATC GGT TTG TTG TA3'		
CMV GT1(gB1)R	5' ATG ACC GCC ACT TTC TTA TC 3'	421	Second round
CMV GT1(gB2)R	5' TTC CGA CTT TGGA AGA CCC AAC 3'	611	
CMV GT1(gB3)R	5'TAG CTC CGG TGT GAA CTC C 3'	190	
CMV GT1(gB4)R	5' ACC ATT CGT TCC GAA GCC GAG GAG TCA 3'	465	
CMV GT1(gB5)R	5' TAC CCT ATC GCT GGA GAA C 3'	139	
Primer CMV Q2F	5' GTT GAT CCA CAC ACC AGG C 3'		

Genomic Viral Extraction

Viral DNA was extracted using QIAamp® virus kits (Qiagen, Germany) according to the manufacturing instruction.

Viral Detection by Real-time PCR

The CMV was detected using real time PCR according to (Peres et al. 2010). The primers and probes sets were 5′ GAAGGTGCAGGTGCCCTG 3′, 5′ TGTCGACGAACGACGTACG 3′, 5′FAM-ACGGTGCTGTAGACCCGCATACAAA-TAMRA3′) respectively and were supplied by the Integrated DNA Technologies company (IDT). The Internal control was ß-actin gene (TaqMan® ß-actin detection reagents - Applied Biosystems, USA). The real-time PCR was performed with a mixture containing: 3 mM MgCl2; 10 µM dATP, dCTP, dGTP, dTTP; 5 U/µl of Platinum Taq (Invitrogen), 60 ng DNA templates, 150 nM of forward and reverse primers (CMVUS17F, CMVUS17R for CMV detection) and 2 µM of the specific Taq Man CMV probe (PE Applied Biosystems). The single PCR was performed under the following conditions: 1 cycle at 50°C for 5 -min, 95°C for 10 min and 40 cycles at (95°C for 20 sec and 60°C for 1 min).

CMV Genotypes Using Nested PCR

The CMV genotypes were determined according to (Tarragó et al. 2003) using the primers sets supplied by the IDT. The primers sequence described in **Table 1**, targeting the glycoprotein B (UL55) gene flanking region, The first and the second rounds of nested PCR amplification were carried out in a total volume of 25 µl of premixed (Intron,) according to the manufacture instruction using 4 µl DNA extract for the first round, the PCR programmed (1 cycle predenaturation for 5min at 95°C and 35 cycles at 94°C for 30 Sec, 60°C for 1 min, and 72°C for 30 Sec, and 1 cycle final extension at 72°C for 10 min). 2 µl PCR product used for a second round with an equimolar mixture primer work solution of 10 pmol of each inner primer (CMVGT1_, CMVGT2_, CMVGT3_, CMVGT4_, CMVGT5_, and CMVQ2+), with same premix as mentioned above. The second PCR round was carried out under the same conditions that was used in the first PCR round except the primer multiplex annealing temperature was 58°C instead of 60°C .The expected nPCR bands size were 421, 611, 190, 465, and 139 bp for CMVGT1 gB1, CMVGT gB2, CMVGT3 gB3, CMVGT4 gB4, and CMVGT5 gB5, respectively. PCR products were electrophoresed according to (Sambrook et al. 1989), on the prepared agarose gel that stained with 3 µl/100ml Red Safe Nucleic Acid (RSNA) staining solution (20,000x), and visualized under 336 nm of UV light. PCR products of amplified gene were sent to Macrogene (Korean Company), where they were sequenced employing the same primers used for the PCR.

RESULT AND DISCUSSION

Active cCMV infection during pregnancy is a major cause of congenital CMV infection in fetus with comparable distribution of different genotypes. In the recent study active CMV infection was detected in 24 samples (12%) in real time PCR with significant Ct value <35. In general the prevalence rates of HCMV was variable depending on IgM and IgG serological result in non-pregnant women which represented 1% , and 84% respectively , and in pregnant women represented 2.5% and 90% (Alwan 2011). In Pakistanian study conducted by (Mujtaba et al. 2016) mentioned to that 20% (82/409) pregnant women were found positive for CMV DNA by PCR Our result was less than what was records by (Pati et al. 2013) in USA that was about 28% of CMV infants infected were symptomatic at birth, and disagree with HCMV IgM positive was 51% in Iraq seroprevalence study on pregnant women (Noor Al- Huda et al. 2016). Molecular screening depends on the presence of the active virus in the bloodstream, which is present in the primary infection, while the HCMV IgM antibodies are formed and persist for an extended period of time after primary infection. This explains the difference in the rates of infection records (Bernard et al. 2015).

The active 20 samples CMV nucleic acid was submitted to CMV genotyping using M-nPCR and sequence techniques. Twelve out of 20 (60%) recipients had CMVGT2 (gB2) genotype; 6 out of 20 (30%), recipients had CMVGT4 (gB4); 2 out of 20(10%), CMVGT1 (gB1). Our finding indicated that the most frequent genotypes were gB2 followed by gB4, showed in **Fig. 1**. Hameed and Aziz (2016) refers in their molecular study that about 22.85% miscarriage women were found to be HCMV positive in Baghdad. Dieamant and others (2013) in their study on Brazilians patients with active CMV infection found that the most frequent genotypes were gB1 and gB2 (74%). it appears that all CMV genotypes are transmissible (Arav-Boger 2015). The most of symptomatic cCMV infections were resulting from the first trimester maternal infection, but the asymptomatic maternal infection in the later second

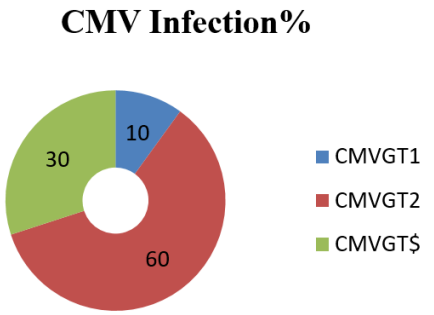

Fig. 1. The Genotypes percentage distribution among CMV infections

and third trimester were associated with a lower rate of sequelae in 85–90% of infected offspring, but is capable of causing hearing defects (Buxmann et al. 2017, Daiminger et al. 2005). Sequence data related to the gB gene on representative strains of the various genotypes were retrieved from NCBI GenBank and included in the sequence alignment and phylogenetic analysis had processed in MEGA 6.

The analysis involved 30 nucleotide sequences with a total of 370 positions in the final dataset. The evolutionary history was deduced using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA)

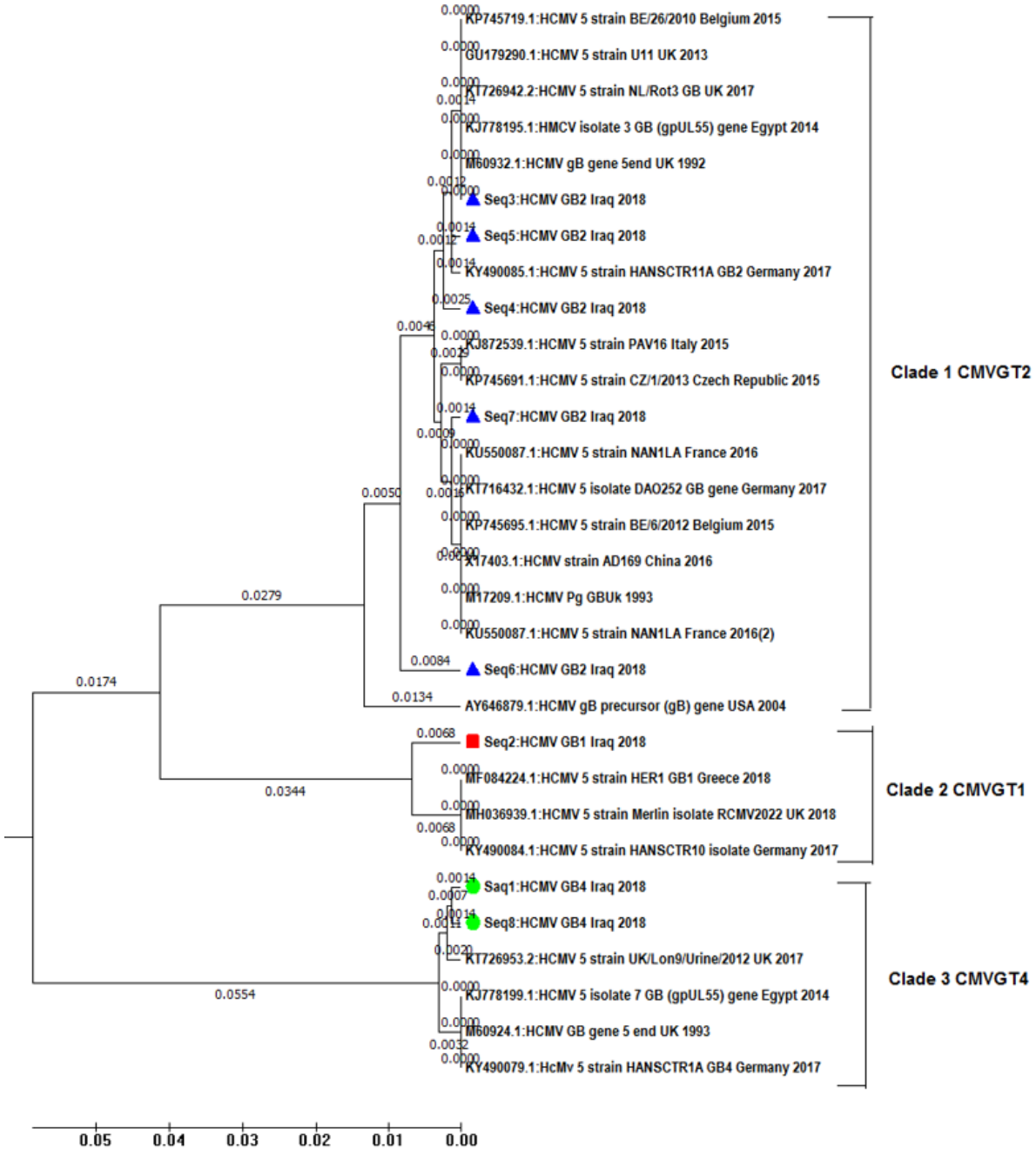

Fig. 2. The phylogenetic tree of CMV partial glycoprotein B (UL55) gene. Iraqi isolates strains (gB2) marked with blue triangles, (gB4) marked with green dots, and (gB1) marked with red square in compared with NCBI sequenced data. Evolutionary history was inferred using the UPGMA method. Evolutionary analyses were conducted in MEGA6 software

method (Sneath and Sokal 1973). The optimal tree with the sum of branch length = 0.20693057 is shown. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004), and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated (Tamura et al. 2013). Moreover the blasted CMV sequences were 99% identical to CMV sequences in NCBI GenBank strains and this agrees with Renzette et al. (2011) who refers to that CMV has a large genome (235 kb) encoding 165 genes and has been shown to be highly genetically diverse with polymorphisms dispersed across the virus genome. Many researchers concluded the relationship of CMV infection with occurrence of some disease, Nahar and others (Nahar et al. 2018) concluded that gB1, gN3, and gH2 gene were the most frequently observed genotypes in ulcerative colitis patients, it might be an association between these genotypes of HCMV and ulcerative colitis, other study in symptomatic and asymptomatic neonates suggested the association between gB1 genotype and hepatic damage. Al-Baiati and others (Al-Baiati et al. 2014, Mirzamasoumzadeh and Mollasadeghi 2013, Rehman 2015, Uçar et al. 2015) refers that PCR results revealed that 89% and 77% of infertile women and 87% and 67% of breast cancer women were positive for UL55, UL97 genes respectively. Our findings emphasize to conduct a comprehensive large scale survey of HCMV infection and introduction of country wide routine screening at maternity clinics for early diagnosis of HCMV to reduce its associated devastating outcomes.

CONCLUSIONS

In conclusion, Due to the absence of Iraqi large scale surveillance studies, and the lack of routine screening tests at maternity clinics, so there is an urgent need for early diagnosis of CMV in our population to reduce congenital CMV infections and to determine the real burden of the CMV infection, also early detection may lead to new antiviral treatment options. Real time PCR assay was very accurate, rapid, and sensitive method of CMV early diagnosis, also M-nPCR and sequencing was a specific assay for CMVGT genotyping.

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