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Cytomegalovirus, molecular and phylogenetic study in woman in Wasit Province- Iraq

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Abstract

Cytomegalovirus (CMV) is a common virus that can cause a wide range of clinical manifestations, depending on the health status of the individual and the specific context of infection and can be transmitted from a pregnant woman to her developing fetus. Congenital CMV infection can lead to a range of birth defects, including hearing loss, vision impairment, developmental delays, and intellectual disabilities. One hundreds blood sample from women who have CMV infection and a 50-blood sample from non-infected women as a control were collected from gynecologists' clinics in Al-Kut city of Wasit province. All the samples were serologically tested, positive samples were molecularly tested by nested PCR and ten samples of them were selected for sequencing. Ninety individuals (90%) tested positive for anti-CMV IgG, while ten individuals (10%) exhibited detectable levels of anti-CMV IgM while the control samples give negative results with both antibodies. The molecular test performed by nested PCR 31 yielded positive results. A high prevalence of CMV antibodies among female participants, suggesting a potential link between CMV infection and female abortion. Molecular assays, known for their speed and utility, hold particular value for diagnosing HCMV. Specifically nested PCR emerged as a valuable tool for detecting CMV infection. The existence of polymorphic CMV strains may indicate the widespread occurrence of CMV infection in women.

Keywords: Cytomegalovirus, nested PCR, serological test, abortion.

Introduction

The Cytomegalovirus (CMV) belongs to the Herpesviridae family of double-stranded DNA viruses. The virus may multiply in endothelial cells, the central nervous system, liver, lungs, and digestive tract during active infection. The virus is present in bodily fluids (urine, saliva, vaginal discharge, semen, breast milk) during original infection and periods of reactivation, making the seropositive individual a reservoir of the virus (1,2). Cytomegalovirus is the most prevalent cause of intrauterine infection. It is also a common cause of loss of sensorineural hearing and mental retardation (3). For fetal infection to happen in pregnant women, viral replication in uterine and placental compartments is likely required the replication of the virus in peripheral bodily fluids, like blood or cervicovaginal secretions, commonly known as 'shedding'. CMV can remain dormant in a number of types, including, fibroblasts, macrophages & endothelial cells (4). Serological testing continues to serve as a viable alternative for diagnosing HCMV infection. These tests are particularly effective in assessing the status of an infection, identifying acute or recent infections through the detection of IgM antibodies, and determining past infections by checking for the presence of HCMV IgG antibodies (5).Human CMV infection is widespread throughout the world. About 60% of adults in developed regions and more than 90% of adults in many developing countries have specific IgG antibodies (6). Nearly all populations tested positive for CMV infections in serological surveys with seropositivity in various regions of the world varying from 40% to 100% (7; 8). The highest CMV seroprevalence was typically seen in South America, Africa, and Asia. However, Cytomegalovirus seroprevalence was also higher in the Middle East (such as Turkey and Palestine) and some countries in Europe (such as Italy and Sweden). The United States and Western Europe have the lowest seroprevalence rates. CMV seroprevalence varied significantly within the United States of America as well, with differences across states of up to 30 percentage points (9). In neonates and adults, the cobas® CMV is suitable for routine diagnostic laboratory investigation of CMV infection (10)

Women having a history of abortion displayed 60.2% IgM seropositivity in different research in the Wasit province of Iraq (11), although this was not significantly distinct from the control group. In women who tested positive for Cytomegalovirus IgM, a substantial percentage of recurrent miscarriage (two & three or more) took place. Since 12% of women of childbearing age in Mosul, Iraq tested positive for the cytomegalovirus, the majority of congenital CMV infections are probably the consequence of maternal reinfection (12).

Polymerase chain reaction (PCR) analysis is a virological detection technique that proves valuable in diagnosing viral infections due to its capacity to identify minute quantities of viral DNA (5). Polymerase chain reaction-based CMV DNA detection provides a sensitive, quick, and precise method of identification. Whole blood and plasma are the most common specimens for HCMV (13)

The study was aimed to detect seroprevalence of IgM and IgG antibodies in women, molecular confirmation of seropositive infections using the conventional PCR assay, phylogenetic analysis of some positive PCR local isolates in the NCBI and association between seropositive infections and some related-risk factors.

Materials and Methods

Blood samples were gathered alongside corresponding data. (age, Genito-urinary problems, miscarriage problems, social status (marriage), gestation status (have pregnancy or not), occupation status (employee, housewife, student), residence (urban, and rural), from gynecologists' clinics in Al-Kut city of Wasit province. The time of collection ranged from January to June 2023. One hundred (100) females experiencing CMV infection, 50 healthy women as a control (healthy), venous blood sample (5-10 ml) was collected from each patient. Each blood sample was divided into two tubes one with gel to obtain serum for serological detection and other with EDTA for molecular detection was stored at -20 C until DNA extraction was performed on it.

Detection of CMV by Cobas E 411

Using the completely automated software-controlled Cobas E 411-Roche immunology analyzer that uses electrochemiluminescence (ECL), a serological test for CMV IgG and IgM antibodies is conducted. It is made to be used for both quantitative and qualitative in vitro analyses employing a wide range of tests.

Nested PCR Amplification:

Extraction of Cytomegalovirus DNA from blood

Initially, For extraction of genomic DNA initially 300 ml of blood according to kit instruction (Wizard® Genomic DNA Purification Kit, Promega, USA). Quality and concentration of template DNA were evaluated by a Nanodrop apparatus (Thermo Scientific, USA). The primer used in this study were published previously by (14). Nested PCR was conducted to confirm the molecular-level identification of CMV by amplifying a region from the envelope glycoprotein gene B (UL55) by utilizing two pairs of primers. The first primers pair sequences gB1 F. (5′CAA-GAR-GTG-AAC-ATG-TCC-GA3′) and gB2 R. (5′GTC-ACG-CAG-CTG-GCC-AG3′) with product size of 520 bp while the second pairs sequences gB3 F. (5′TGG-AAC-TGG-AAC-GTT-TG-GC3′) and gB4 R. (5′GAA-ACG-CGC-GGC-AAT-CGG3′) product 305 bp (Macrogen, Korea).

Amplification of PCR products

A total volume of 25 µl mixture contain 13.5 µl master Mix (Promega, USA) 1 µl of both forward and reverse primers 10 picomole, 2 µl DNA and 8.5 µl distilled water. The mixture vortex for 5 second and subjected to the thermocycler for amplification. The amplification cycle

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began with denaturation at 95^0 C for 10 minutes, then followed by 35 cycles each cycle consists initial denaturation at 95^0 C for 1 minute, annealing at 66.3^0 C for 1 minute and elongation at 72^0 C for 1 minute followed by final elongation at 72^0 C for 7 minutes.
For outer-nested PCR, the PCR mixture with a total reaction volume of 25 μl, containing 13.5 µl master Mix, 1 µl of both forward and reverse primers 10 picomole, 2 µl (PCR product from first step) as DNA templet, and 8.5 µl distilled water. The total volume was subjected for amplification to an initial denaturation step at 95°C for 10 minutes. DNA was amplified for 35 cycles as follows: denaturation at 95°C for 30 seconds, primer annealing at 63.8°C for 45 seconds, and followed by a step of elongation at 72 for °C 30 seconds; the final elongation was at 72°C for 7 minutes. (14). Annealing temperatures were adjusted using www.thermofisher.com software. Agarose gel electrophoresis 1% used to analyzed 8 µl PCR product at 100 volts for a duration of 45 minutes.

Statistical Analysis

Microsoft Excel 2016 was used to evaluate data analysis. Frequency and percentages were used to express category variables. The value of the relationship among categorical variables was assessed by Chi-square. $P<0.05$ was regarded as statistically significant.

Results

Prevalence of Anti-*CMV* IgG and IgM Antibodies in Women

This study enrolled 150 women, among whom 100 tested positive for CMV infection. The majority of these infected women were pregnant, accounting for 90% of cases. The participants had an average age of 24 years, with an overall age range of 15 to 41. As shown in Table (1), half of the women in the study fell between the ages of 26 and 35. The remaining 50 individuals tested negative for CMV. Their serological analysis, performed using the Cobas e 411 system, showed no presence of IgM antibodies. This finding suggests they had no recent infection. However, the detection of IgG antibodies in these subjects indicates a prior exposure to the virus or a potential reinfection at an earlier time. Overall, among the 100 CMV-positive cases, IgG antibodies were detected in 90 subjects (90%), while IgM antibodies were found in 10 subjects (10%).

Table (1): Categorization of Study Participants Based on Sociodemographic Traits

Variables		Frequency	Percent%	IgG	IgM	P-value
Age category	<25 years	42	42%	36	6	0.374
	26-35 years	50	50%	46	4	
	>36 years	8	8%	8	0	
	Total	100	100%	90	10	
Residence	Rural	27	27%	25	2	
	Urban	73	73%	65	8	
	Total	100	100%			
Occupation	employee	20	20%	16	4	0.204
	Housewife	74	74%	68	6	
	Student	6	6%	6	0	
	Total	100	100%			
Gestation Status	Pregnant	90	90%	80	10	0.27
	Non-Pregnant	10	10%	10	0	
	Total	100	100%			
Urogenital problems	Yes	20	20%	20	0	0.096
	No	80	80%	70	10	
	Total	100	100%			
Miscarriage problems	Yes	24	24%	24	0	0.00
	No	76	76%	66	10	
	Total	100	100%			

Tab* p-value < 0.05 significant

In terms of residency, the majority of the study population (72%) lived in the urban setting of Al-kut city. Statistical analysis revealed that the presence of CMV IgG or IgM antibodies showed no significant correlation with the patients' area of residence. Analysis of the connection between CMV antibodies and urogenital problems revealed no statistically significant difference in sero-reactivity among the women (p=0.096). Similarly, the association between CMV antibodies and patient occupation status. Here, too, no statistically significant difference was found in the sero-reactivity of IgG or IgM antibodies (p=0.204). In contrast, a statistically significant difference was observed in the association of CMV antibodies (IgG and IgM) with miscarriage history (p=0.00). This finding is presented in Table (1).

***CMV* Molecular Detection**

The analysis of PCR data involved detecting amplified gene bands on a 1% agarose gel. The objective was to amplify the HCMV UL55 (gB) gene from whole blood samples. Electrophoresis of the PCR products revealed that bands observed at the expected length of 305 bp, corresponding to the UL55 gene fragment of the envelope glycoprotein, were identified as positive. Based on this molecular analysis of the 100 samples examined, 31 (31%) tested positive for the HCMV UL55 gene, while 69 (69%) tested negative.

DNA Sequencing

Following the successful amplification of the glycoprotein gene, high-quality PCR products were selected for sequencing. Seven of the ten chosen samples were sequenced successfully, while the remaining three did not yield adequate sequence data. All obtained sequences were verified for accuracy by comparing them to reference sequences in the gene database using a BLAST search. Subsequently, these sequences were aligned using multiple sequence alignment software, such as BioEdit or MEGA, to identify any genetic variations within the gB gene.

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DNA Alteration

BLAST nucleotide analysis showed that five of the seven sequenced isolates shared 98.31% identity with CMV gB reference sequences from Germany (accessions MT044484.1, MT044478.1) and the United Kingdom (MT044482.1, MT044481.1). The remaining two isolates showed 100% identity with the CMV gB reference sequence MT044476.1 from the United Kingdom.

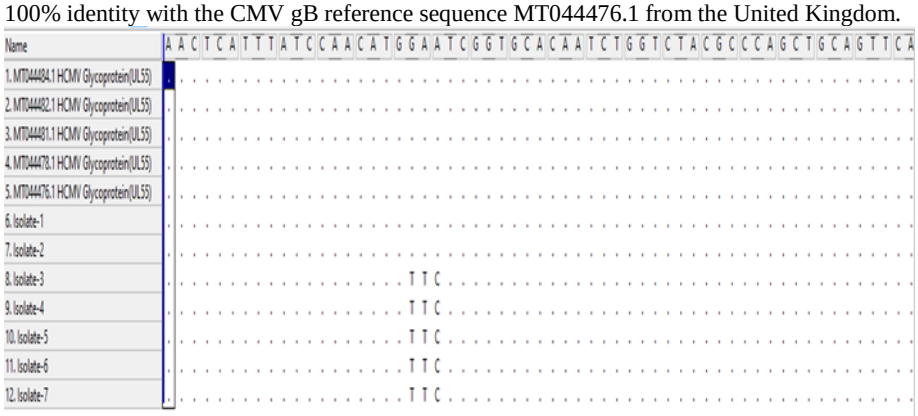

Figure (1): The *CMV* glycoprotein gene several sequence alignment in BioEdit using the GenBank *CMV* reference strain, shows that 5 *CMV* isolates have transversion mutations.

Following sequence alignment, three of the isolates were found to carry a transversion mutation, where the nucleotide triplet **GAA** in the reference sequence (MT044848.1) was replaced by **TTC** (Figure 1). This mutation resulted in an amino acid substitution from glutamic acid (E) to phenylalanine (F) (Figure 2).Finally, phylogenetic analysis based on glycoprotein B gene (UL55) of the HCMV confirmed a genetic correlation between the isolates sequences. The constructed phylogenetic tree revealed that first cluster included HCMV strains from Germany and the United Kingdom, including sources from whole blood and plasma samples, which grouped together from moderate to high bootstrap percentage between 57–100%. In contrast, all Iraqi isolates were separated from the Germany and the United Kingdom strains. This Iraqi cluster demonstrated high bootstrap values (100%), indicating strong genetic close among the Iraqi. These findings presented of geographically relationship among HCMV isolates in this study (Figure 3).

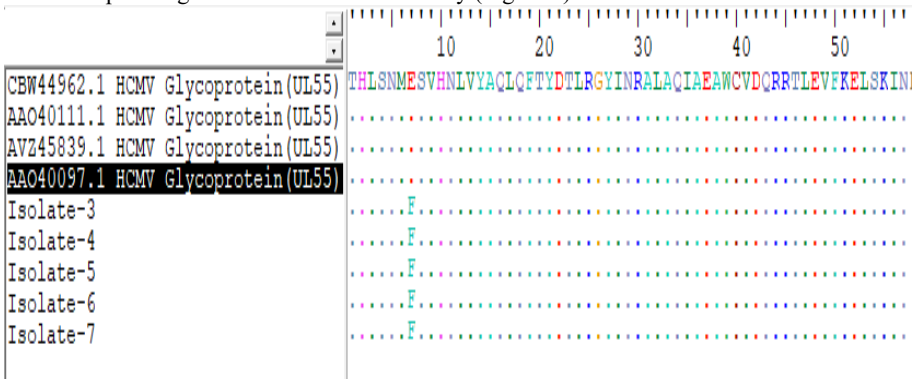

Figure (2): Comparing the gB gene's amino acid several sequence alignments to those of other gB genes in the database. the amino acid Substitution from Glutamic acid(E) to Phenylalanine (F)

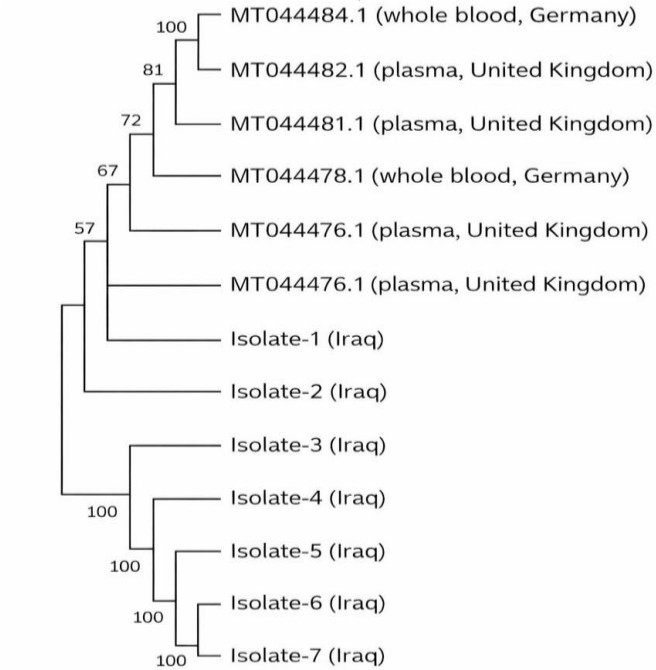

Figure (3): Phylogenetic tree constructed from the gB gene sequences of 7 women CMV isolates. The MEGA neighbor-joining method was used to build the phylogenetic tree analysis

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Discussion

Serological Analysis

Cytomegalovirus (CMV) is one of the world's most prevalent viruses and can lead to serious illness with numerous complications. In this study, the Cobas CMV IgG test yielded positive results for 90% of the women (90 out of 100 cases). This high seroprevalence suggests that the majority of participants had been exposed to CMV at some point in their lives. Following exposure, CMV IgG antibodies typically circulate in the blood for life, which may offer significant long-term protection against future infections. Conversely, a negative CMV IgG result indicates no prior infection with the virus (15). The IgG seroprevalence observed in our study aligns with findings from other regions, including Nigeria 97.2%, (16), Sudan 95%, (17), and China 96%, (18). A worldwide estimate of CMV seroprevalence shows that it affects 83% of the general population, 86% of women in their reproductive years, and 86% of blood or organ donors. Among these groups, the highest seroprevalence is recorded in the Eastern Mediterranean region as defined by the World Health Organization (WHO) at 90%, while the lowest is found in the WHO European region at 66% (6).In the current study, ten women (10%) tested positive for CMV IgM antibodies using the Cobas test. The presence of IgM typically indicates a recent active infection or a reactivation of the virus (19). This finding is consistent with the 7.2% IgM seroprevalence reported by in Malaysia (20).The age of participants, which ranged from 14 to 41 years, was another factor analyzed in this study. Given this restricted range, participants were divided into three age groups. The group over 26 years of age showed the lowest conception rate. Consistent with existing literature, the research indicates that susceptibility to CMV infection increases with age. Notably, however, 62% of the CMV infections identified in pregnant participants occurred in individuals under 30 years old, suggesting that associated risk factors are also influenced by socioeconomic status. (21; 22). Regarding occupation, the majority of pregnant participants (80%) were housewives, with the remainder being employed or students. In terms of clinical outcomes, the present study found that pregnant women with active CMV infection had a 24% risk of abortion. This finding aligns with other research indicating significant risks from active infection, such as a 30% chance of mother-to-child transmission leading to congenital infection (23). Infections are estimated to be responsible for approximately 5% of unintended abortions. Among the microbiological causes, viruses play a significant role in recurrent cases, often due to their ability to establish chronic or recurring infections within the female reproductive system. Cytomegalovirus (CMV) is of particular concern, as it is a recognized cause of congenital infection. Primary CMV infection during pregnancy carries a substantial risk of vertical transmission to the fetus (24).The dynamics of CMV in pregnancy are complex. Unlike some other pathogens, the virus can reactivate during a woman's childbearing years. Consequently, it can be transmitted to the embryo even in the presence of pre-existing maternal antibodies, making management more challenging. Furthermore, a community's socioeconomic status is a well-documented risk factor influencing CMV seroprevalence (25).Geographic differences in seroprevalence may be explained, in part, by the predominant routes of transmission. In rural areas, the virus is believed to spread primarily via saliva in early childhood, often linked to conditions of poor sanitation. In contrast, sexual transmission appears to be the main route of infection for women of reproductive age in urban settings (26).Regarding residency, our finding that living in an urban area independently predicted CMV IgG seropositivity aligns with some reports (27), (28) but contrasts with others (29). Finally, while typical pathological signs of CMV are often found in renal tissue, several studies indicate only minimal supporting evidence for an association between CMV infection and an increased risk of significant renal system malformations or clinical disease (30).

Molecular and Phylogenetic Analysis

The discrepancy in reported congenital CMV infections between serological and PCR tests likely stems from differences in the sensitivity and specificity of the two methods. An ideal diagnostic test must be highly sensitive to detect infection before the onset of serious clinical disease, and sufficiently specific to rule out false-positive results. In this context, the detection of HCMV DNA via PCR offers greater sensitivity and specificity than serological assays like the Cobas test, making it a more reliable tool. For instance, a positive IgM result alongside a negative PCR finding can occur. This may be due to the persistence of IgM antibodies long after the initial infection in some asymptomatic individuals, where the viral load has fallen below the detectable threshold of the PCR assay

Phylogenetic Analysis

The glycoprotein B (gB) gene, which encodes a crucial envelope protein of HCMV, is directly involved in viral entry, cell-to-cell transmission, and the fusion of infected cells. As the primary target for neutralizing antibodies, it also plays a significant role in activating the host's immune response. Consequently, gB antibodies are a major focus of research for their therapeutic potential in viral neutralization (31). Mutations within the gB gene, particularly transversions that alter the amino acid sequence, can enhance viral fitness and adaptability (32). In this study, such transversion mutations were identified in five Iraqi gB strains, which are associated with increased viral pathogenicity. Phylogenetic analysis revealed that the HCMV strains from this study cluster with diverse global strains from regions such as Germany and the United States, indicating a broad international circulation rather than a geographically isolated lineage. The presence of these globally circulating strains in Iraqi women with primary infection or active disease suggests significant local and international transmission of these HCMV variants.

Discussion of Molecular and Phylogenetic Results

The molecular detection of HCMV by PCR targeting the UL55 (glycoprotein B/gB) gene was a specific method to detect active infection. Notably, 31% (31 of 100) of the samples in this cohort tested positive for HCMV DNA, an expected detection range for viremia in clinical studies, and highlights the relevance of PCR over serology to ascertain active replication [31, 32]. Successful amplification of the 305 bp gB fragment was the template that gave rise to further genetic characterization. The profiling of high-quality amplicons from seven isolates provided detailed genomic analysis. Compared with worldwide circulating strains, BLAST comparisons indicated high levels of homology. Five isolates were identified with reference sequences from Germany and the UK at 98.31%, whereas two were identified with a UK reference at 100% (MT044476.1). In our study identified this pattern, we can conclude that the HCMV strains are not isolated local variants but rather belong to large, international clades, which is in line with the fact that the gB gene is also conserved but suggesting some form of global strain exchange [33]. The critical finding was the identification of one particular transversion mutation (GAA→TTC) in three isolates (Figure 1), leading to a non-conservative amino acid substitution from glutamic acid to phenylalanine (E→F) (Figure 2). Notably, mutations to the gB gene within the immunogenic domain are of interest because they could impact viral entry, tropism and immune escape [34]. The radical change in amino acid properties between the E→F substitution (charged acidic to hydrophobic aromatic) could modify the conformation and antigenicity of the protein. Such non-synonymous mutations in gB are associated with improved viral fitness in vivo, and may be relevant to vaccine design because gB is an important target for neutralising antibodies [35, 36]. Phylogenetic analysis (Figure 3) provided a highly visible confirmation of the genetic relationships suggested by BLAST. The cluster of Iraqi isolates in association with European strains is consistent with the concept of an integrated transmission network rather than localized regional evolution of the strains. This phylogenetic closeness to well-characterized global reference sites offers a template to interpret the lineage of these local strains in terms of evolution and suggests that pathogenicity, or drug resistance characteristics may also possibly become relevant locally [37]. Taken together, this analysis of the PCR detection, sequencing, and phylogenetic analysis demonstrated the identification of local HCMV gB strains of interest. The identification of particular, potentially severe amino acid mutations in the isolated isolates demonstrates the evolution of the virus. Further, phylogenetic correlation with European strains emphasizes the global circulation of HCMV genotypes. These findings add to the molecular epidemiology of HCMV in the region and offer genetic information for further research assessing the clinical burden of these specific gB mutations.

Conclusion:

Study results indicate a significant association between CMV infection and miscarriage, alongside identifying genetic variations in the viral strains present. High sero-prevalence of CMV antibodies observed in female participants. CMV infection may significantly contribute to female abortion. Molecular assays are valuable for HCMV diagnosis, especially for vulnerable groups. Nested PCR with gB primers effectively identifies CMV infection. Presence of polymorphic CMV strains indicates widespread infection in women.

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Authors' Declaration Conflicts of Interest:
The authors have no conflict of interest to disclose.

Authors' contributions:
Study design by Tahmasebi P. analyzed by Abdul Saheb Z.A. and Al-badrawi T.Y.G. acquired the data, and all authors approved the final text after their contributions to interpretation and correction were made.

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