Genetic modifications within *TLR4* and *TLR9* genes contribute into congenital toxoplasmosis and cytomegaly development

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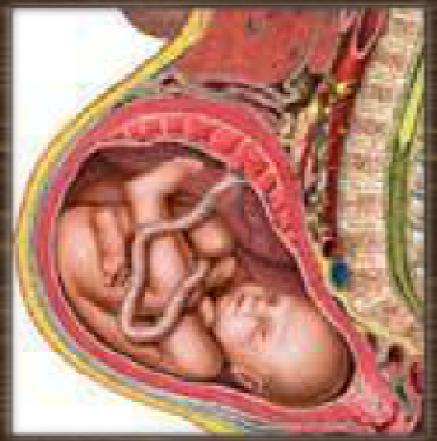

3rd International Conference on Clinical Microbiology and Microbial Genomics

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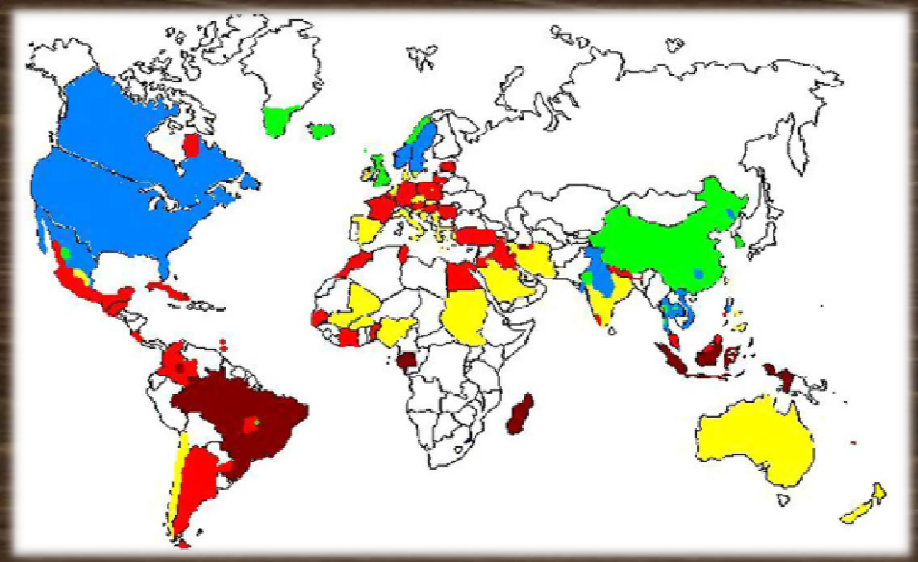
T. gondii and HCMV infections within pregnancy

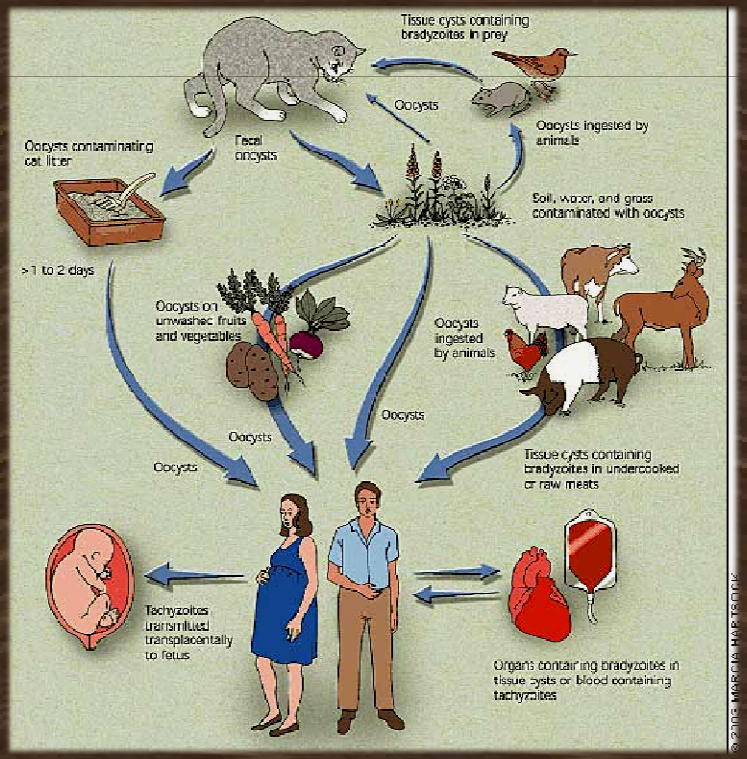
Common cause of intrauterine infections

***T. gondii* seroprevalence between 4% and 100% with values over 60% in Central and South America, Africa and Asia**

HCMV prevalence between 40% and 100% dependent on the continents and countries

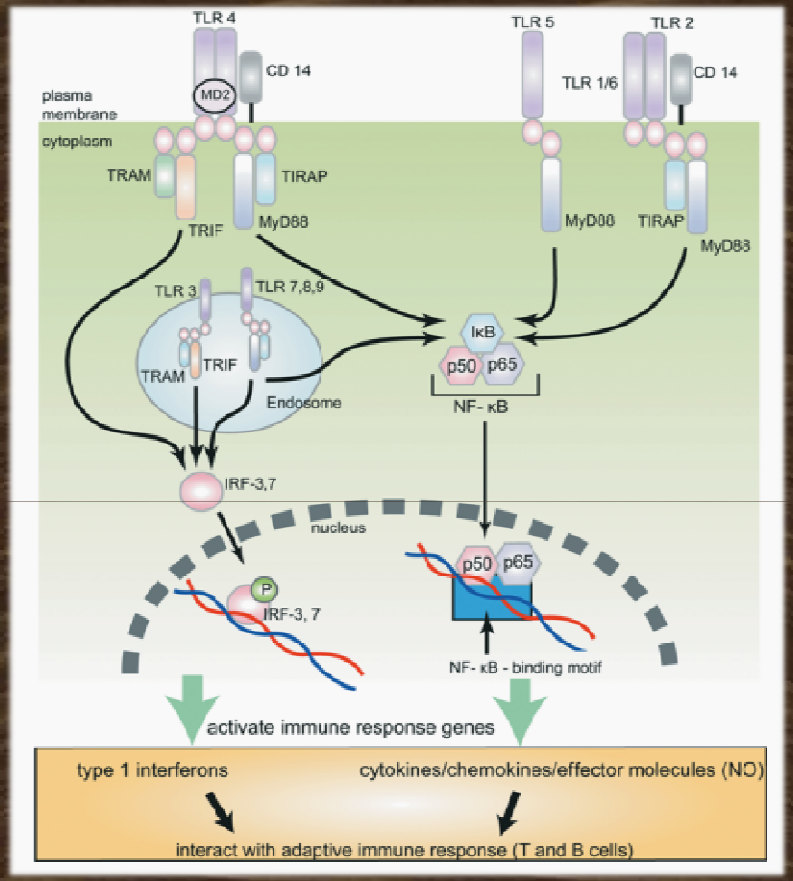


Pappas G. (2009) *Int J Parasitol.* 39: 1385-1394



http://scienceray.com/biology/the-parasite-toxoplasma-gondii/

Role of TLRs in immune response



Misch EA and Hawn TR. (2008) *Clin Sci.* 114(5):347-60

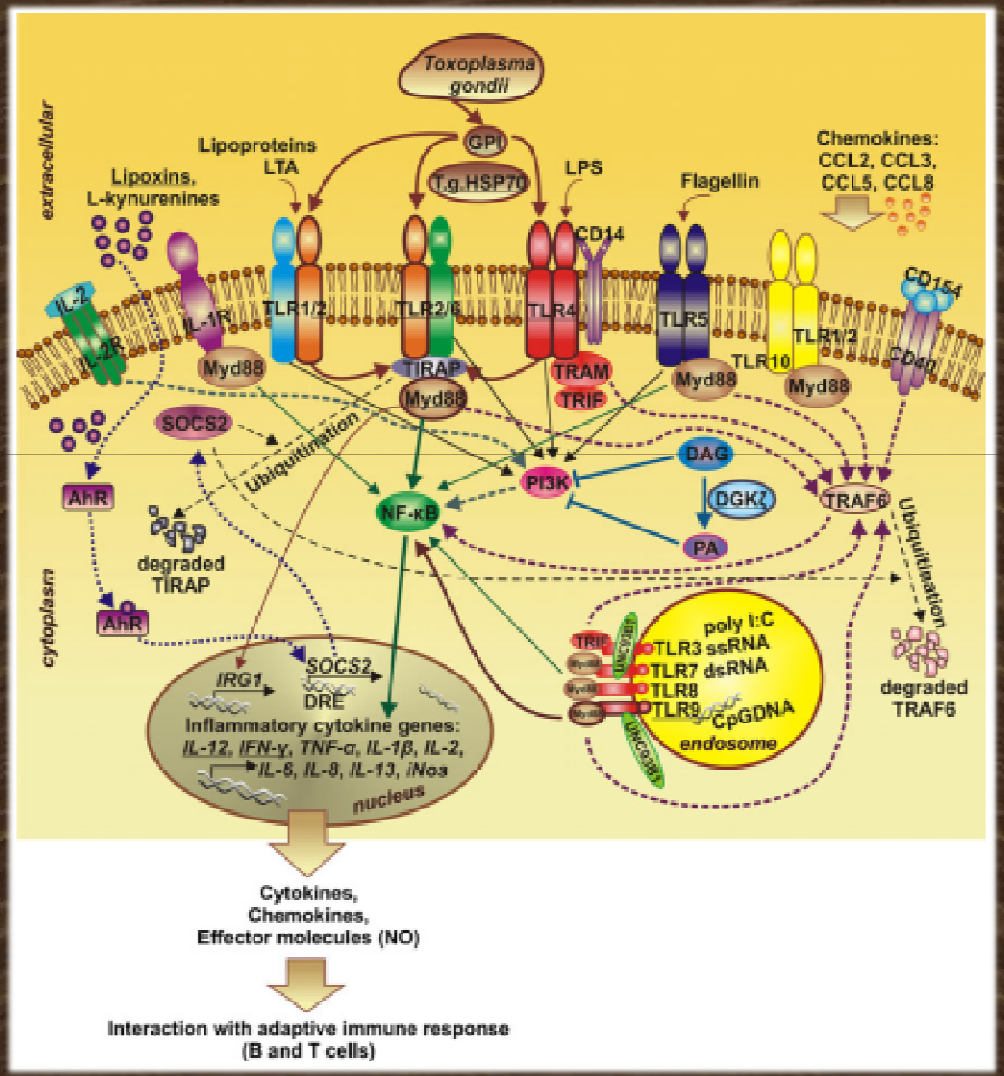
Transduction of signals from PAMPs to the cell interior, activation of these cells and the first line of host defense against pathogens

TLR	Ligands	Origin of Ligand	Possible Role in Disease
TLR1	Triacyl lipopeptides	Mycobacteria	
TLR2	Peptidoglycan	Gram positive bacteria	Sepsis, RA, IBD
	Lipotechoic acid	Gram positive bacteria	
	GPI-linked proteins	Trypanosomes	
	Atypical LPS	Gram negative bacteria	
	Lipoproteins	Mycobacteria	
	Zymosan	Fungi	
	Heat shock protein 70	Host	
TLR3	dsRNA	Viruses	
TLR4	LPS	Gram negative bacteria	Sepsis, RA, IBD
	Fusion protein	RSV	
	HSP 60?	Host	
	Fibrinogen fragments?	Host	
TLR5	Flagellin	Bacteria	IBD, Legionnaire's
TLR6	Diacyl lipopeptides	Mycobacteria	
	Zymosan	Fungi	
TLR7	ssRNA	Viruses	
	Imiquimod, R848	Synthetic	
	Loxiribine	Synthetic	
TLR8	ssRNA	Viruses	
	R848	Synthetic	
TLR9	CpG DNA	Bacteria and viruses	
	Herpes virus DNA	Virus	
	CpG ODNs	Synthetic	
TLR10	Not determined		

Ho J et al. (2004) *Tannaffos*. *3*(11):7-14

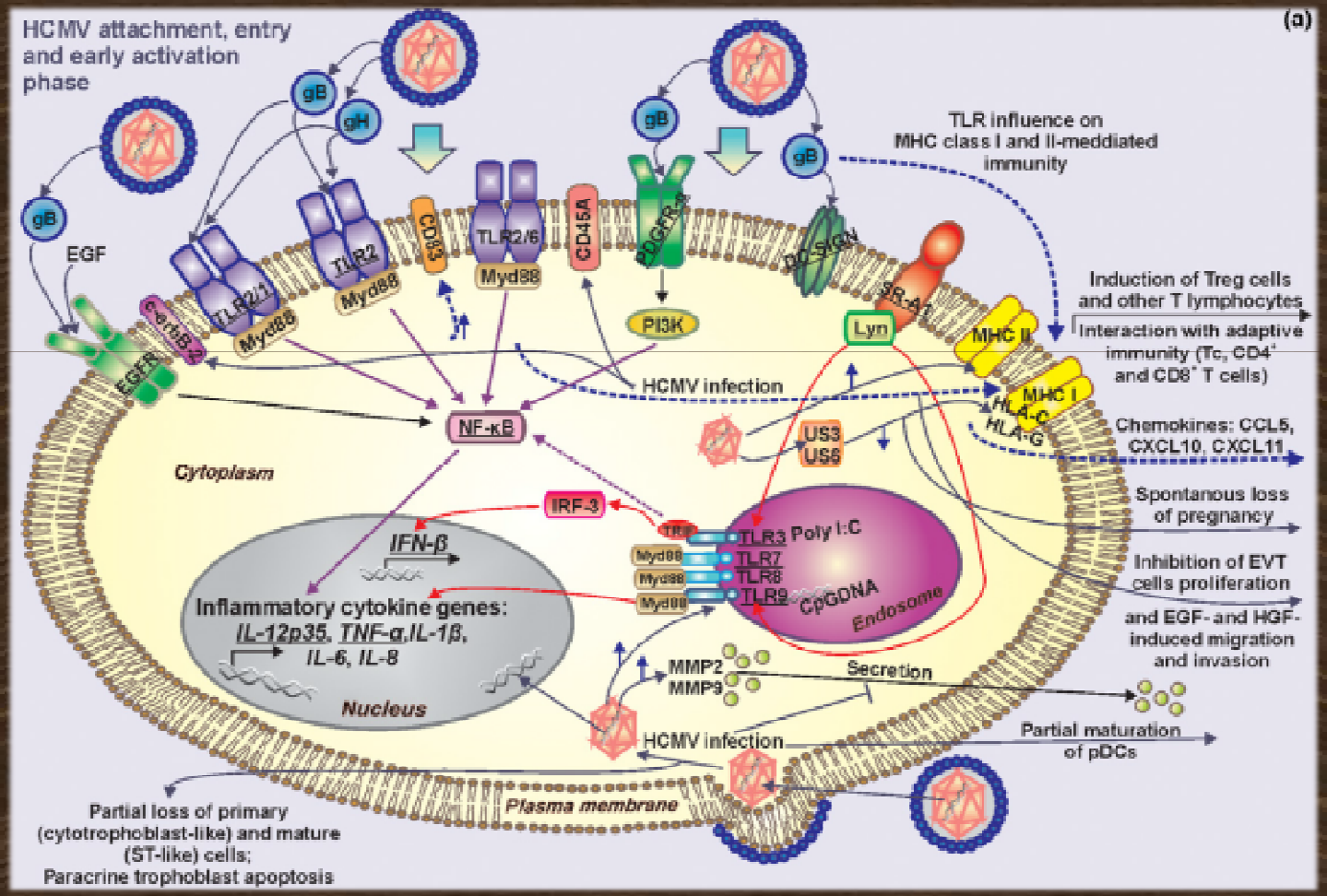
Important molecules activating and inducing both innate and adaptive immune response

Contribution of TLR2, TLR4 and TLR9 in the immunity against *T. gondii*



Wujcicka W et al. (2013) *Eur J Clin Microbiol Infect Dis.* 32(4): 503-511

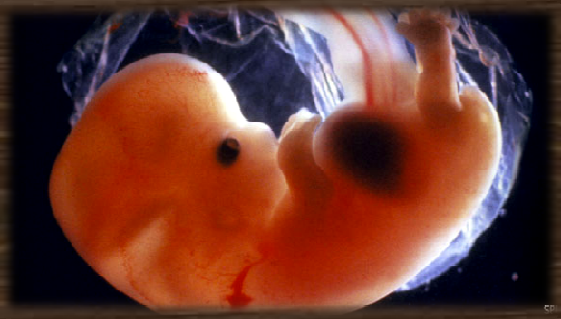
TLRs activity in the immune response against HCMV



Wujcicka W et al. (2014) *Pathog Dis.* 70(1):3-16

Aims of study:

- Determination of a distribution of genotypes at *TLR4* and *TLR9* polymorphic sites in fetuses and newborns congenitally infected with *T. gondii*
- Comparison of the genotypic profiles at *TLR* SNPs between the offsprings with congenital toxoplasmosis and cytomegaly

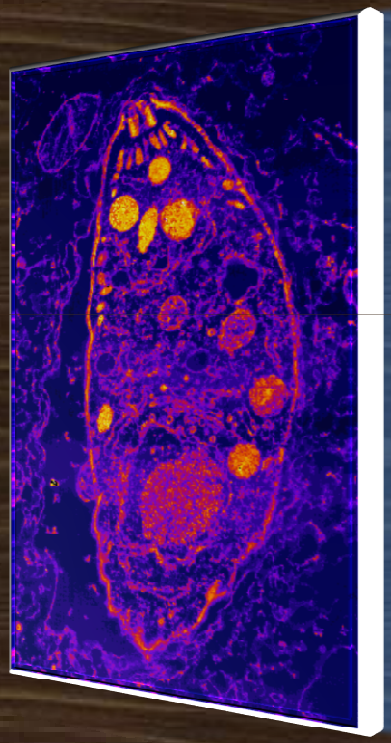


Materials and Methods: Collection of clinical specimens from fetuses and newborns

Eighteen (18) fetuses and newborns with congenital toxoplasmosis and 41 control cases without *T. gondii* intrauterine infection

Samples collected retrospectively (15 *T. gondii* infected cases and 23 controls) and prospectively (three *T. gondii* infected cases and 18 controls)

Fifteen (15) fetuses and newborns with HCMV infection and 18 control cases of HCMV-seronegative status



http://protoplasmix.wordpress.com/tag/toxoplasma-gondii/

Classification of clinical specimens for molecular studies

Serological screening:

Screening for *T. gondii* IgG and IgM antibodies as well as IgG avidity performed with an enzyme-linked fluorescent assay (ELFA) (Vidas Toxo IgG II; IgM; or IgG Avidity, bioMérieux, France)

HCMV screening with Eti-Cytok G-Plus and Eti-Cytok M-Reverse Plus tests (Diasorin/Biomedica, Italy) used between 2000 and 2001, VIDAS CMV IgG and IgM tests (bioMérieux, France) between 2001 and 2006, anti-CMV IgG and IgM tests (Diasorin/Biomedica, Italy) between 2006 and 2011 years and ELFA assays from 2012 year

Clinical symptoms observed in pregnant women and their fetuses:

Flu-like symptoms in mothers

Ultrasound markers in fetuses with toxoplasmosis:

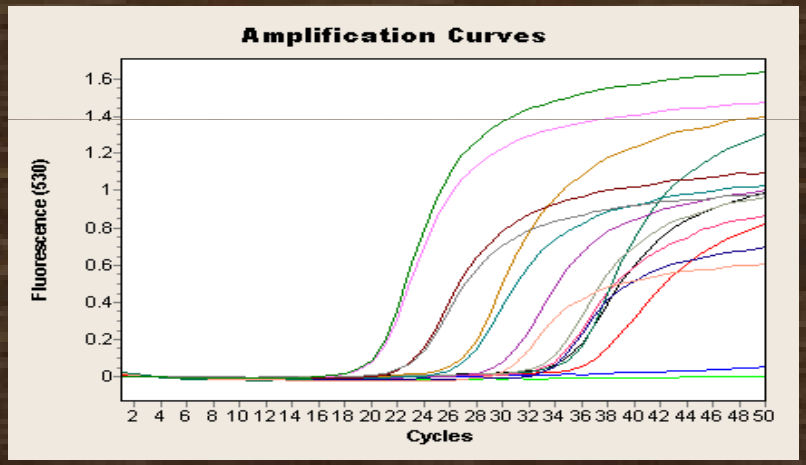
hydrocephalus, chorioretinitis, cerebral calcification and stroke, as well as microcephaly, hepatosplenomegaly, fetal hydrops and IUGR

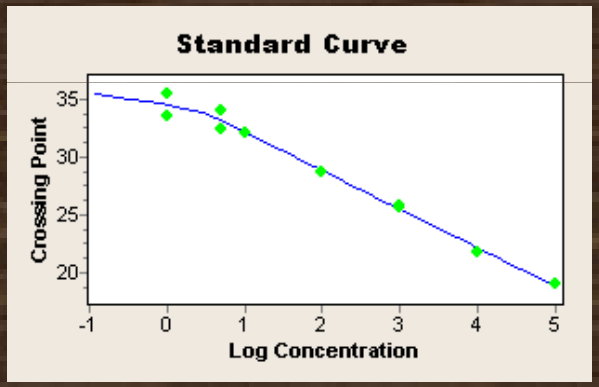
Ultrasound markers in fetuses with cytomegaly:

ventriculomegaly, hydrocephalus and fetal hydrops as well as IUGR, ascites, pericardial effusion, cardiomegaly and the presence of hyperechogenic foci in different organs like the fetal brain, liver and pancreas

Detection and quantification of *T. gondii* and HCMV DNA

Locus Gene	Sequences of primers and probe (5' →3')	GenBank	Annealing temperature (°C)	PCR product (bp)
AF 179871 B1	CAAGCAGCGTATTGTCGAGTAGAT GCGTCTCTTTCATTCCCACATTTT 6-FAM- CAGAAAGGAACTGCATCCGTT-NFQ	AF 179871	60	83





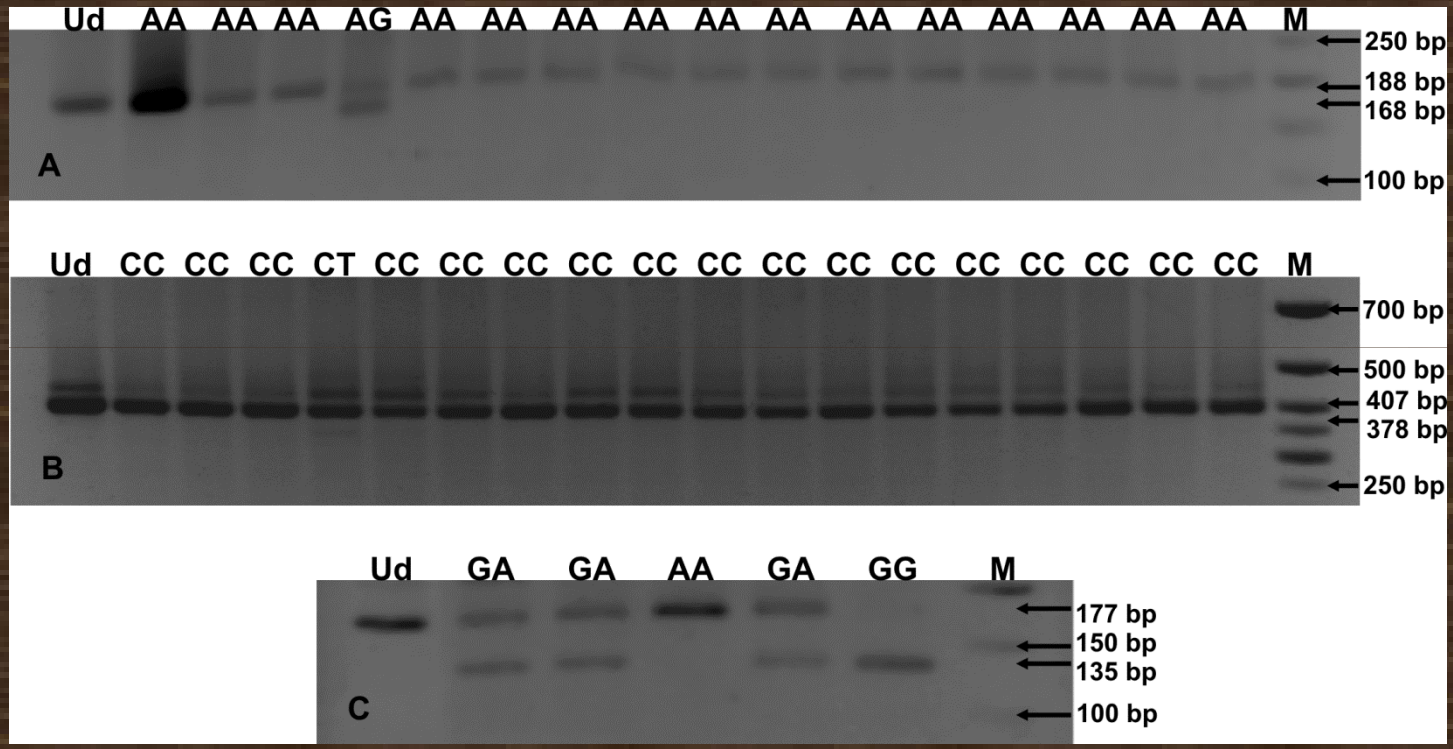
Amplification of HCMV *UL55* gene fragments of 150 bp using primers and probes of the following sequences: 5'-GAGGACAACGAAATCCTGTTGGGCA-3', 5'-TCGACGGTGGAGATACTGCTGAGG-3', and 5'-6-FAM-CAATCATGCGTTTGAAGAGGTAGTCCA-TAMRA-3'

Genotyping of SNPs located at *TLR4* and *TLR9* genes

Gene	SNP name		Primer sequences (5'-3')	Annealing temperature [°C]	Amplicon length (bps)	Restriction enzyme	Profile (bps)
TLR4	896 A>G (rs4986790)	External	For: AAAACTTGTATTCAAGGTCTGGC	52	355	NcoI	AA: 188 AG: 188, 168, 20 GG: 168, 20
			Rev: TGTTGGAAGTGAAAGTAAGCCT				
		Internal	For: AGCATACTTAGACTACTACCTCCATG	61	188		
			Rev: AGAAGATTTGAGTTTCAATGTGGG				
	1196 C>T (rs4986791)	External	For: AGTTGATCTACCAAGCCTTGAGT	52	510	HinfI	CC: 407 CT: 407, 378, 29 TT: 378, 29
			Rev: GGAAACGTATCCAATGAAAAGA				
		Internal	For: GGTTGCTGTTCTCAAAGTGATTTTGGGAGAA	59	407		
			Rev: ACCTGAAGACTGGAGAGTGAGTTAAATGCT				
TLR9	1635 G>A (rs352140)	External	For: GTCAATGGCTCCCAGTTCC	52	292	BstUI	GG: 135, 42 GA: 177, 135, 42 AA: 177
			Rev: CATTGCCGCTGAAGTCCA				
		Internal	For: AAGCTGGACCTCTACCACGA	59	177		
			Rev: TTGGCTGTGGATGTTGTT				

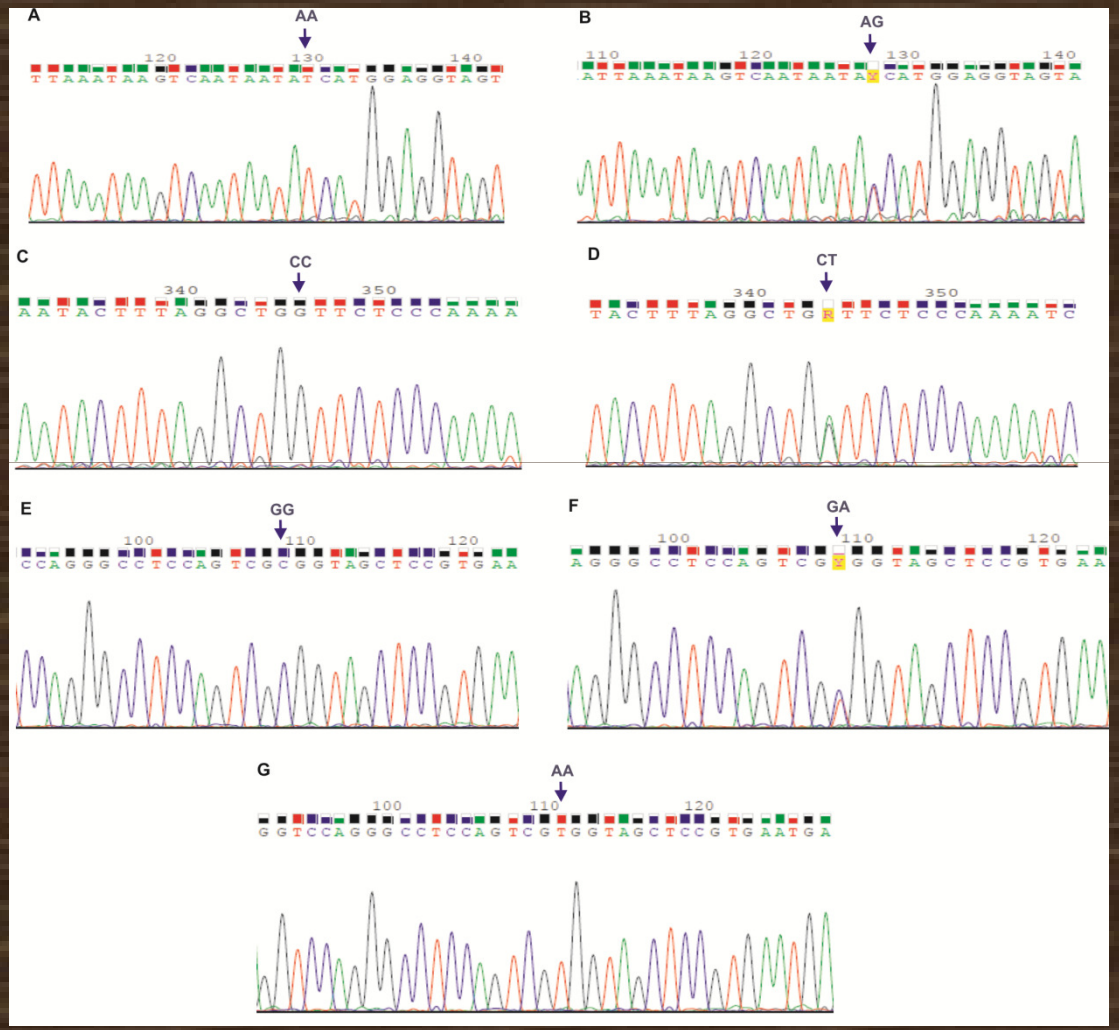
Sequencing of randomly selected PCR products for distinct genotypes at *TLR4* 896 A>G, *TLR4* 1196 C>T and *TLR9* 1635 G>A SNPs

Results: Products of multiplex nested PCR-RFLP analysis of *TLR4* and *TLR9* SNPs



Agarose gel electrophoresis of PCR-RFLP products for profiling of genotypes at *TLR4* 896 A>G SNP (A), *TLR4* 1196 C>T SNP (B) and *TLR9* 1635 G>A SNP (C)

Sequencing of the selected amplicons for *TLR4* and *TLR9* SNPs



Chromatograms for DNA fragments encompassing *TLR4* 896 A>G SNP (A, B), *TLR4* 1196 C>T SNP (C, D) and *TLR9* 1635 G>A SNP (E-G)

Relationship between *TLR* polymorphisms and congenital toxoplasmosis

Gene polymorphism	Genetic model	Genotype	Genotype frequencies; n (%)[a]		OR[b] (95% CI)[c]	*P*-value[d]
			Infected cases	Seronegative controls		
TLR4 896 A>G		AA	17 (94.4%)	19 (95%)	1.00	
	---	AG	1 (5.6%)	1 (5%)	1.12 (0.06-19.28)	0.94
TLR4 1196 C>T		CC	17 (94.4%)	18 (90%)	1.00	
	---	CT	1 (5.6%)	2 (10%)	0.53 (0.04-6.39)	0.61
TLR9 1635 G>A		AA	3 (16.7%)	8 (40%)	1.00	
		GA	11 (61.1%)	10 (50%)	2.93 (0.60-14.23)	
	Codominant	GG	4 (22.2%)	2 (10%)	5.33 (0.62-45.99)	0.230
		AA	3 (16.7%)	8 (40%)	1.00	
	Dominant	GA-GG	15 (83.3%)	12 (60%)	3.33 (0.72-15.37)	0.110
		AA-GA	14 (77.8%)	18 (90%)	1.00	
	Recessive	GG	4 (22.2%)	2 (10%)	2.57 (0.41-16.12)	0.300
		AA-GG	7 (38.9%)	10 (50%)	1.00	
	Overdominant	GA	11 (61.1%)	10 (50%)	1.57 (0.43-5.71)	0.490
	Log-additive	---	---	---	2.40 (0.83-6.95)	0.090

[a] n, number of tested fetuses and newborns; [b] OR, odds ratio; [c] 95% CI, confidence interval; [d] logistic regression model; $P \leq 0.050$ is considered as significant

Frequencies of alleles at *TLR4* and *TLR9* SNPs

Gene polymorphism		No.[a] of carriers with *TLR* alleles (%)		*P*-value[b]
		Congenital toxoplasmosis	Seronegative control	
TLR4 **896 A>G**				
Alleles	A	35 (97.2)	39 (97.5)	0.940
	G	1 (2.8)	1 (2.5)	
TLR4 **1196 C>T**				
Alleles	C	35 (97.2)	38 (95.0)	0.619
	T	1 (2.8)	2 (5.0)	
TLR9 **1635 G>A**				
Alleles	G	19 (52.8%)	14 (35.0%)	0.118
	A	17 (47.2%)	26 (65.0%)	

[a] No., number; [b] Pearson's Chi-squared test; P ≤ 0.050 is considered as significant

Genotypic profiles at *TLR4* and *TLR9* SNPs in congenital toxoplasmosis and cytomegaly

Significantly less frequent GC haplotype at *TLR4* SNPs in congenital toxoplasmosis than in cytomegaly ($P \leq 0.0001$)

GC haplotype at *TLR4* SNPs and multiple GCG genotypes at *TLR4* and *TLR9* SNPs significantly more frequent in congenitally infected than control cases ($P \leq 0.0001$)

Conclusions

Genetic modifications within *TLR4* and *TLR9* genes might contribute to congenital toxoplasmosis and cytomegaly

Thank You for Your attention!