

AN EGYPTIAN STUDY OF MOTHER TO CHILD TRANSMISSION OF HEPATITIS C VIRUS

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ABSTRACT

This study aims to assess markers of hepatitis C Virus (HCV) including HCV antigen detection in peripheral blood mononuclear cell (PBMNC) lysates, antibodies to HCV core, NS₃, NS₄ and NS₅ epitopes by enzyme linked immunoassay (ELISA) in mothers and their infants using serum samples for both mothers and infants plus saliva for infants, in addition to detection of serum HCV- RNA (viremia) by reverse transcription polymerase chain reaction (RT-PCR). Also detection of anti-HIV IgG class antibodies in mothers' sera. The study sample included 61 pairs of 20-40 years old Egyptian mothers and their infants whose ages ranged from one day to 12 months. All the deliveries were vaginal. Neither the infants nor the mothers had a history of high risk exposure to percutaneous virus transmission. Commercial ELISA kits for IgG, IgM, IgA anti-HCV epitopes and RT-PCR kits were used according to kit instructions. Dot ELISA for detection of HCV antigen in infants PBMNC lysates was an in house test. Previous exposure to HCV infection indicated by antibodies to the HCV six epitopes was detected in 8/61 (13%) mothers. Active HCV infection by anti-core and HCV-RNA detection was 5/8 (62.5%). All mother samples were negative for HIV antibodies. The infants born to those 8 mothers included four {4/8(50%)} having serum IgG antibodies to HCV epitopes. None of these four infants had saliva IgM anti-HCV and one had saliva IgA antibodies to HCV epitopes excluding NS₅. The PBMNC lysates belonging to these eight infants contained HCV-antigen in two samples {2/8(25%)}, and these two infants had serum

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HCV-RNA (i.e viremia). In our HCV markers assessment the detection of HCV RNA in mothers' sera as well as anti-core and anti-NS 3 was informative of HCV infectious potential of the mother. PBMNC lysate for HCV antigen detection or saliva sample instead of serum for antibody / RNA detection were not sensitive. Antibodies detected in the 7 days to 6 months old infants were passively transmitted maternal antibodies as there was no anti-HCV in the 6-12 months old infants' sera. There are two limitations in this small sized study: one is the unknown HCV-RNA load in viremic mothers, the second is the duration to study mother and infant pairs over 1-2 years.

Keywords: Egyptian; Hepatitis C virus; Mother to child; Transmission

INTRODUCTION

HCV causes, hardly diagnosed acute hepatitis infection (Houghton, 2009) which goes on to chronic liver disease (CLD) with cirrhosis that can end into hepatocellular carcinoma (HCC). The World Health Organization (WHO) declared HCV a global health problem (World hepatitis alliance, 2009). The seroprevalence rate of HCV in Egypt is about 15% with HCV RNA in 10 % (El Zanaty and Way, 2009). There are at least 7 HCV genotypes and more than 50 subtypes (Kuiken and Simmonds, 2009). The progress from asymptomatic acute hepatitis to CLD and to HCC, as well as, the response to available combination therapy of pegylated interferon and ribavirin is genotype related. Ray

et al. (2000) reported the dominance of genotype 4 in Egypt. Another study of Egyptian HCV genotype 4 reported that it was extremely RNA sequences variable while the functional and immunological determinants of both cytotoxic-T-lymphocyte and neutralization epitopes varied widely (Genoves *et al.*, 2005). All these factors make the prediction of liver disease; serum HCV RNA load and the degree of HCV infection of PBMNC very difficult. Mother to infant transmission of HCV is controlled by serum viral load (HCV-RNA greater than one million copies / ml), maternal PBMNC HCV infection (Azzari *et al.*, 2000), coinfection with human immune deficiency virus (HIV) (Moriya *et al.*, 1995; Zanettie *et al.*, 1995; Paccagnini *et al.*, 1995;

Sabatino *et al.*, 1996; Tovo *et al.*, 1997 and Thomas *et al.*, 1998) or practicing injection drug use (Spencer *et al.*, 1997; Resti *et al.*, 2002; and Paez Jimenez *et al.*, 2009). Thorough evaluation of risk factors in mothers to child transmission of HCV in a study of 21,791 pregnant Japanese women stressed that maternal HCV-RNA high viral load was the highest factor; then vaginal delivery and a low titer anti-NS₄ antibody (Okamoto *et al.*, 2000). In 1994-1996 a study of 499 pregnant Egyptian females residing in United Arab Emirates; showed that 65 (13%) of these mothers had anti-HCV while 20 (4%) had HCV RNA with 20/20 (100%) mother to infant transmission of HCV RNA at birth and 65/65 (100%) transplacental anti- HCV transfer. By the fifteenth months of age there was a 5% mother to infant asymptomatic HCV vertical transmission (kumar *et al.*, 1997). We report a study of 61 mother-infant pairs for markers of HCV infection transmission. Infant blood, saliva and maternal blood were used. We compared the detection of HCV-RNA with anti HCV epitope specific antibody plus PBMNC HCV antigen detection.

SUBJECTS, MATERIALS & METHODS

Subjects:

Sixty one pairs of Egyptian parturient mothers 20-40 years old and their infants whose ages ranged from one day to one month (15 infants), one to three months (15 infants); three to six months (15 infants) and six to twelve months (16 infants) were the subjects in this study. Informed oral consents were taken from the mothers as self and as guardians of the infants. All infants were delivered vaginally and breast fed. All mothers were healthy without any history of high risk practice and or exposure to risk of percutaneous transmission of blood borne viruses.

Samples:

Three to five milliliter (ml) venous blood was collected from the mothers and their infants in a phenol free heparinized tube. Three to five milliliter saliva was collected from each infant in a saliva collection container containing 0.1 % sodium azide (0.1 ml / 5 ml saliva).

Separation of infant's peripheral blood mononuclear cells (PBMNC):

Three to five ml of the anti-coagulated infant's blood samples was layered on 2.5 ml of the ficoll hypaque density gradient (Boyum, 1974). Samples were centrifuged for 30 min at 1500 rpm and 20°C. The PBMNC interface was carefully aspirated and washed twice by centrifugation at 1500 rpm for 10 min. with Hank's balanced salt solution (HBSS) (Boyum, 1974). The PBMNC were lysed by three cycles' freeze-thaw and the lysate was stored at - 80°C. The supernatant plasma / platelet layer of the gradients was converted to serum by adding 0.1 ml 2 molar solution of Ca Cl₂ / 0.9 ml plasma and incubation at 37°C for 2 hours followed by centrifugation at 3000 rpm for 10 minutes then the serum was aliquoted and stored frozen at - 80°C.

ELISA tests:

Detection of anti-HCV antibodies:

Murex VK4 (Wellcozyme anti-HCV) second generation ELISA kits were used according to the manufacturer instructions for the detection of anti-HCV IgA, IgM, and IgG class antibodies against all

HCV epitopes and the specific IgG anti-HCV core, NS₃, NS₄ and NS₅ epitopes using maternal / or infant blood and infant saliva samples with modifications. A peroxidase labelled mouse monoclonal antibody to human IgG or alkaline phosphatase labeled goat antihuman IgM, or goat antihuman IgA peroxidase labeled (Sigma) were used in the final step of ELISA with alternative choices of an enzyme substrate for the final colour reaction to produce soluble or insoluble end product.

Detection of HIV antibodies:

Abbot second generation ELISA kit was used according to the manufacturer instructions for detection of anti-HIV IgG class antibodies in mothers serum samples.

Detection of HCV antigen in infants PBMNC lysates by Dot ELISA:

In brief lymphocytes were lysed by five times freeze and thaw cycles. A nitrocellulose (NC) membrane sheet was wetted in distilled water for 15 minutes, then transferred into a manifold apparatus and was subjected to vacuum to create "cups" in the pliable NC sheet. The cups were filled with 2 µl of lymphocytes lysate (three

cups/sample), followed by incubation in a humid chamber for 30 minutes at room temperature (RT), then washed twice with pH 7.4 phosphate buffer saline Tween 20 (0.3) (PBS T20). A 2 µl of human anti-HCV positive serum was applied to one cup / every sample and 2 µl of human serum negative for anti-HCV in one cup / sample. In the third cup / sample 2 µl of blocking buffer was applied. Following this the NC sheet was incubated for 30 minutes at RT, then washed 3 times with pH 7.4 PBS T20 buffer and 2 µl of mouse monoclonal antihuman IgG horseradish peroxidase conjugated serum (Murex) was applied to each well and the NC sheet was incubated for 30 minutes at RT then washed 5 times with pH 7.4 PBS T20 buffer. The NC sheet was transferred to a tray to be soaked in freshly prepared enzyme substrate solution {3, 3' Diaminobenzidine tetra hydrochloride (DAB)} and incubated at RT for 20 minutes in the dark. The NC sheet was washed by dist. H₂O followed by air drying and photographed. A dot of brown precipitate developed in the wells wherever there was positive dot ELISA in the first row only.

Detection of HCV viremia:

RNA extraction:

Total RNA was extracted from sera by silica as described by **Boom *et al.* (1990)**.

HCV primers:

The primers were selected from the highly conserved 5'-UTR of HCV genome (**Choo *et al.*, 1989**). The anti-sense primer for c-DNA synthesis was HCV- 6 nucleotides (NT) 5'-ACC-TCC-3' (319-324). The primers used for PCR were RB6A and RB6B for amplification of 265 bp of the 5'-UTR, RB6A 5'-GTG AGG AAC TAC TGT CTT CAC G-3' {NT 47-68} and RB6B 5'-ACT CGC AAG CAC CCT ATC AGG- 3' {NT. 292-312} (**Zekri *et al.*, 1995**).

HCV-cDNA synthesis:

RT reaction was performed in a 25 µl reaction volume containing: 20 U of RNAase inhibitor (Promega Biotec Madison, WI, USA), 67 mM Tris-HCl (pH 8.8), 17 mM ammonium sulphate, 1 mM β mercaptoethanol, 6 mM EDTA (pH 8.0), bovine serum albumin (Boehringer) 0.2 mg/ml, 6 mM MgCl₂, 25 ng of primer HCV-6, 0.6 µl of 25mM (each) deoxynucleoside triphosphate, 11.5 µl of the extracted RNA and 200 U of superscript-II RNAase-H reverse transcriptase (RTASE) (Gibco-BRL, Gaithersburg, MD, USA).

The mixture was incubated at room temperature for 5 min and then at 42°C for 60 min then RTASE was denatured by incubation for 5 min at 95°C.

HCV-RNA detection by PCR:

The PCR was performed in a 50 µl volume containing 2.5 U of Taq polymerase (Perkin- Elmer Cetus), 50 mM Tris HCl (pH 8.3), 20 mM KCl, 1.5 mM MgCl₂, bovine serum albumin (BSA) 1 mg/ml, 12.5 µl of the RT reaction mixture, 200 µM (each) deoxynucleoside triphosphate and 100 ng each of primers RB-6A and RB-6B. HCV cDNA samples were denatured at 95°C for 5 min then were subjected to 35 cycles of amplification in a DNA thermal cycler (Type 480: Perkin-Elmer Cetus). Each cycle consisted of denaturation for 1 min at 95°C, annealing for 1 min at 55°C, and extension for 2 min at 72°C. The last cycle extension was for 10 min at 72°C. Upon completion of the amplification reaction, 10 µl of each RT-PCR product was analyzed by electrophoresis through a 1.2% agarose gel in Tris-Acetate- EDTA buffer (pH 8.0) with 0.2% ethidium bromide. The size of the HCV amplicon compared with DNA molecular size ladder and a band with 266 bp was considered a positive HCV RNA (**Figure 2**).

RESULTS

HIV antibodies:

All mothers' sera were negative for IgG antibodies against HIV

HCV markers:

Table (1) shows the epitope specific IgG antibody pattern of mothers-infants-pair sera. Positive detection of IgG anti-HCV points to passive transfer of maternal IgG anti-HCV to their infants with IgG anti- HCV survival till the infants six month age. **Table (2)** shows comparison between infant's serum/saliva anti- HCV epitopes reactivities. Saliva IgA anti-HCV epitopes was detected in one six month old infant who had serum HCV-RNA as well as PBMNC lysate HCV antigens; all are suggestive of active HCV infection in spite of serum IgG anti- HCV (see **Table 1**). Another infant had serum HCV-RNA and PBMNC lysate HCV antigens suggestive of HCV replication without detectable saliva IgA anti-HCV epitopes. These data may be markers of an early phase of infant HCV infection. Our results at large do not support the use of saliva for anti-HCV antibody assessment during probable early stage of mother to infant HCV infection-transmission from anti HCV seropositive mothers. **Figure (1)** illustrates paired mother-infant

HCV markers. The presence of IgG anti- HCV total proteins; anti-core, anti-NS and anti-NS₅ in mothers' sera was also detectable in infants' sera. Meanwhile anti-NS₄ may be detectable in the maternal but not in the infant serum suggesting selective passage of anti-epitopes. Serum HCV-RNA was detected in two infants aged three and six months but was not detected in the

other three infants (7 days; 3 months; 12 months) despite that all the mothers had HCV-RNA in their sera. These finding show that mother-infant HCV RNA transmission incidence was {2/5(40%)}, an information that has to be relayed to anxious HCV viremic mothers/parents to relief guilt feelings associated with this social problem.

Table 1. Serum Hepatitis C Virus Epitope Specific IgG Antibody and HCV-RNA Pattern of Egyptian Parturient Mothers versus their Infants.

Subject No.	Mothers infants Pairs	Age of infant	IgG Anti- HCV (all epitopes)	IgG				Serum HCV-RNA
				Anti-core	Anti-NS ₃	Anti-NS ₄	Anti-NS ₅	
1	Mother		+	+	+	+	-	+
	Infant	7 days	+	+	+	+	-	-
2	Mother		+	+	+	-	+	+
	Infant	3 months	+	+	+	-	+	+
3	Mother		+	+	+	+	-	+
	Infant	3 months	+	+	+	+	-	-
4	Mother		+	+	+	+	-	+
	Infant	6 months	+	+	+	+	-	+
5	Mother		+	-	-	-	-	-
	Infant	6 months	-	-	-	-	-	-
6	Mother		+	-	-	-	-	-
	Infant	10 months	-	-	-	-	-	-
7	Mother		+	-	-	-	-	-
	Infant	10 months	-	-	-	-	-	-
8	Mother		+	+	+	+	-	+
	Infant	12 months	-	-	-	-	-	-

+ Detectable IgG antibody or HCV-RNA - Undetectable IgG antibody or HCV-RNA

Table 2. Correlation of HCV markers in infants borne to IgG anti-HCV positive Egyptian parturient mothers: Infants serum IgG anti- HCV, HCV-RNA, Lymphocyte lysate HCV antigen and saliva IgA anti-HCV.

Infant age	Infant Serum		Infant Saliva								PBMNC lysate HCV antigen	
	IGg Anti-HCV (all epitopes)	HCV-RNA	Anti-core		Anti-NS ₃		Anti-NS ₄		Anti- NS ₅			
			IgM	IgA	IgM	IgA	IgM	IgA	IgM	IgA		
7 days	+	-	-	-	-	-	-	-	-	-	-	-
3 months	+	+	-	-	-	-	-	-	-	-	-	+
3 months	+	-	-	-	-	-	-	-	-	-	-	-
6 months	+	+	-	+	-	+	-	+	-	-	-	+
6 months	-	-	-	-	-	-	-	-	-	-	-	-
10 months	-	-	-	-	-	-	-	-	-	-	-	-
10 months	-	-	-	-	-	-	-	-	-	-	-	-
12 months	-	-	-	-	-	-	-	-	-	-	-	-

+ Detectable antibody or HCV-RNA or HCV antigen
 - Undetectable antibody or HCV-RNA or HCV antigen

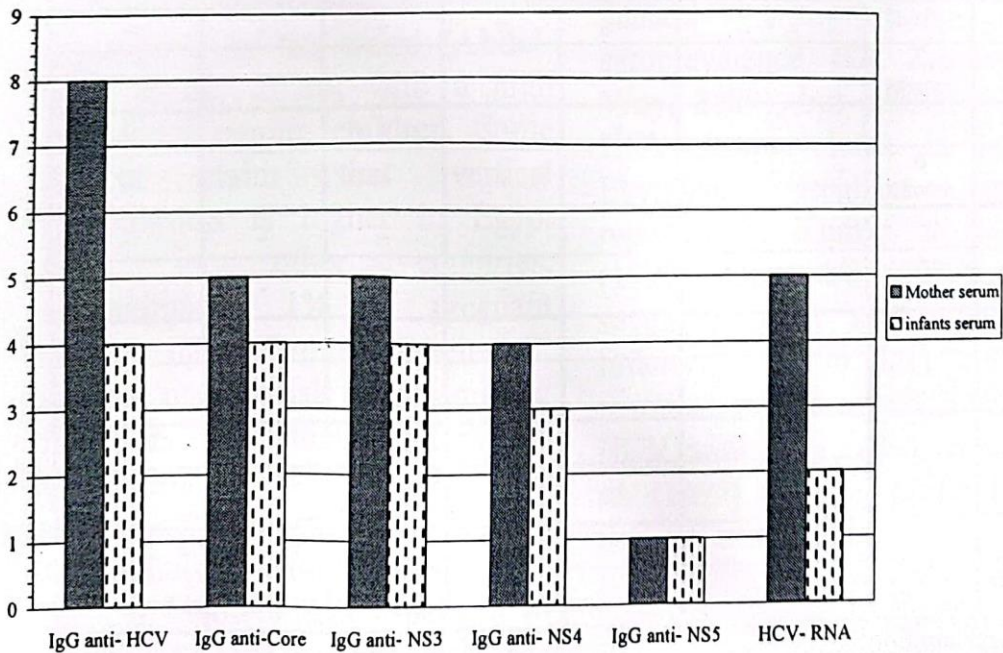


Figure 1. Egyptian Parturient Mother to Infant HCV Infection - Transmission Markers.

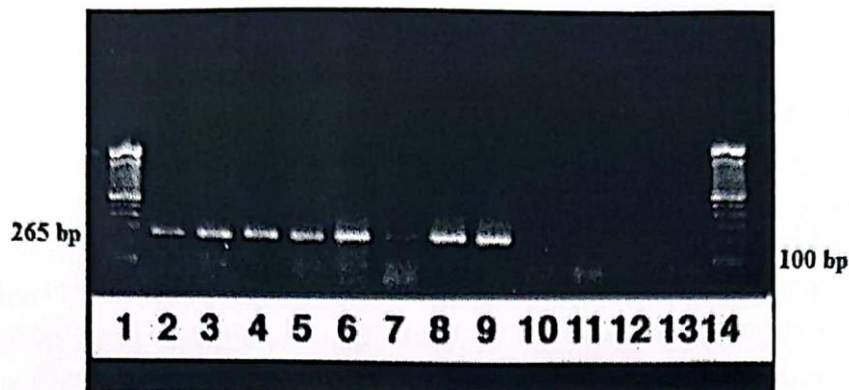


Figure 2. Ethidium bromide stained gel electrophoresis of HCV RT-PCR product. Lanes (1 and 14) are molecular DNA 100 base pair ladder, (lanes 3-9) show HCV-RNA positive serum samples for five mothers and two infants (265 bp), (lanes 10-12) the three HCV-RNA negative infant serum samples. Lanes 2 and 13 showing reference HCV-RNA positive & negative controls respectively.

DISCUSSION

Egypt is considered one of the countries with the highest prevalence of HCV predominantly genotype 4 in the world (**Abdel-Aziz et al., 2000**), with a high prevalence among children. Some reports claim that vertical transmission is higher in Egypt than in other countries, approximately 1% of pregnant women have chronic hepatitis C, and the overall risk of transmitting infection to their infant is approximately 4% to 7% per pregnancy. Transmission appears more likely if the concentration of HCV in blood is high, especially in the third trimester (**Kassem et al., 2000**).

In this study the seroprevalence of IgG anti-HCV in parturient Egyptian mothers was 8/ 61 (13.1%) which is less than the general population 15% seroprevalence (**El Zanaty and Way, 2009**), but similar to Anti-HCV seroprevalence in the 499 Egyptian pregnant mothers reported in Kumar et al study (1997). Only 4/8 (50%) of the seven days to six months old infants borne to IgG anti-HCV reactive mothers had IgG anti-HCV antibodies. Also, **Kumar et al. (1997)** reported 65/65 (100%) passive transmission of IgG anti-HCV antibodies. In our study four infants whose ages were from 6-12 months had undetectable anti HCV-

IgG antibodies indicating early loss or low concentration of maternal anti-HCV beyond detection by commercial kits in apparent absence of markers of infants active HCV without infant active formation of antibodies. The anti-core, anti-NS₃ activity was similar in mothers and infant sera, while anti-NS₅ was lacking in maternal and infant sera in 3/4 pairs and 1/4 of these pairs lacked anti-NS₄ antibodies. These data suggest selective passage of anti-HCV epitope antibodies. Further analysis of specific anti-epitope compartmentalization may clarify some HCV biological activity.

Viremia (HCV-RNA) was detected in 5/8 (62.5%) of anti-HCV seropositive mothers who also had serum anti-core antibody. Two of their infants were HCV-RNA positive (2/5, 40%). One of these infant aged 3 months probably acquired perinatal transmission-infection while the other one was 6 months old at the time of sampling making exclusion of an early infection unrelated to maternal HCV transmission impossible. Also the presence of secretory IgA in this infant saliva sample supports the probability of induced active immune response. The non viraemic anti-HCV seropositive infants also

had serum anti-core antibody, their age at the time of sampling were different. One aged 7 days which was so early to exclude acquired infection as sero-conversion may occur at older age. The other one was 6 months old with the probability of low HCV viral load of his mother. All studies indicate that the higher the concentration of maternal serum HCV RNA the more likely is mother-to-infant transmission. The age of anti-HCV seronegative infant born to viraemic mother was one year at the time of sampling with the probability of complete viral clearance or low titer of HCV-RNA viraemia below the detectable level.

Ceci *et al.* (2001) studied 2,447 HIV negative pregnant women and reported that 78 women were identified as anti-HCV positive and these mother-child pairs were monitored for 2 years; 60 women were found to be HCV RNA positive. Eight infants (13.3%) were identified as infected with HCV. At 2 years old, however, only 2 (3.3%) infants were still positive for HCV RNA. Mother-to-infant transmission correlated with high maternal viral load.

HCV-RNA detection by RT-PCR depends on the sensitivity of the assay. The test we employed

detects one hundred copies / μ l, thus a lower HCV-RNA load whether in the maternal or infant serum will be missed. On the other hand in our study; 5/61(8.3%) of the mothers had HCV-RNA which is more than the 7.8% reported for the general female population and more than the average 5.45% for 20-40 years female (El Zanaty and Way, 2009). The risk of maternal infant-transmission in our study is high 2/5 (40%). These alarming findings should be validated by a large nationwide study to evaluate the parturient mother HCV-RNA status and the outcome of mother-child transmission.

AbdulQawi *et al.* (2010) found that out of 1224 pregnant Egyptian women, 105 (8.6%) were positive for HCV antibody. Only 83 (6.8%) were positive for HCV-RNA. Out of 53 infants tested at first month, 43 (81%) were positive for HCV antibody, but only 7 (13%) were positive for HCV-RNA. After 6 months, only 2 (3.8%) remained positive for HCV RNA which was lower than reported in Egypt before including our study. Their lower prevalence may be because that their study group contained a smaller proportion of parenteral anti-schistosoma therapy treated cohort than did the studies

conducted more than 10 years ago, which may explain the lower prevalence of HCV.

From our study we conclude that ELISA anti-HCV core can be used to assess viremia at a lower cost than RT-PCR. PBMNC lysate for HCV antigen detection or saliva sample like instead of serum for antibody / RNA detection were not sensitive. We recommended a quantitative HCV-RNA assay as an optimum test for HCV-RNA transmission risk assessment of viremic parturient Egyptian mothers. Also recommendations for the management of delivery and possible antiviral administration should be implemented to control this risk factor till an HCV vaccine becomes a reality.

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