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Can HCV RNA Be Detected in Saliva of Egyptian Children Receiving Frequent Blood Transfusions?

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HEPATITIS C virus (HCV) infection is considered a major public health problem all over the world, especially in Egypt. Blood is almost the only route for HCV diagnosis. It has been reported that HCV could be detected in body fluids including saliva which represents an easier route than blood especially in infants and children. This study aimed to: 1) Assess the prevalence of HCV infection among high risk group of Egyptian children. 2) Evaluate the detection of HCV antibodies (anti-HCV) and HCV RNA in saliva against their detection in serum among HCV positive children. *Patients and methods*: this study included 200 children (92 males and 108 females) who were attendants of Haematology Clinic at Abu El-Reish Hospital, Faculty of Medicine, Cairo University, they were receiving frequent blood transfusions during their treatment. Serum and saliva samples were analyzed for detection of anti-HCV by ELISA technique and for HCV RNA by a home made RT-PCR method. Liver function tests were performed also. Results of serum samples revealed that 134/200 (67%) children were anti-HCV seropositive, out of them 79/134 (59%) children had HCV RNA in their sera. Saliva samples of HCV infected children (n=79) showed that 53/79 (67.1%) and 31/79 (39.2%) were anti -HCV and HCV RNA positive, respectively. Prevalence of HCV infection was 39.5% of 200 studied children. We can conclude that: 1) Prevalence of HCV infection among the studied children is considered high. 2) Saliva could play a possible role of biological fluids as a non parenteral route of intrafamilial spread of HCV infection. 3) More sensitive techniques could be developed to use saliva as a reliable route for HCV detection.

Keywords: HCV, PCR and Saliva.

DNA is a polymer of deoxyribonucleoside monophosphates covalently linked by 3'--› 5' phosphodiester bonds. DNA exists as a double-stranded molecule in which the two strands wind around each other, forming a double helix stabilized by H-bonding between bases in adjacent strands[(1)] . In the Watson-Crick model, the bases are in the interior of the helix aligned at a nearly 90 degree angle

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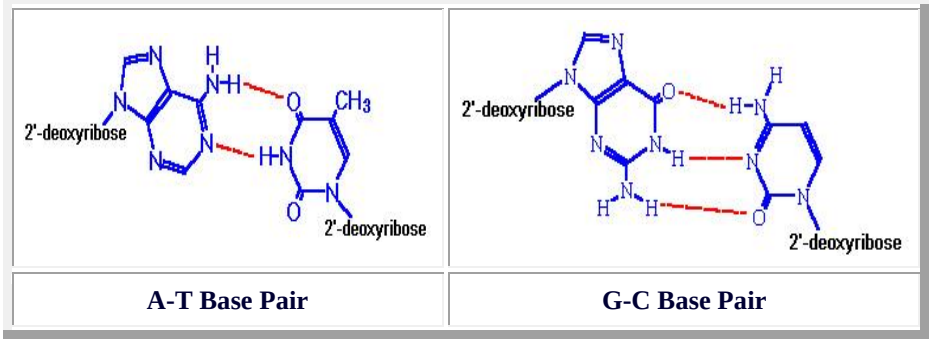
relative to the axis of the helix. Purine bases form hydrogen bonds with pyrimidines, in the crucial phenomenon of base pairing. Experimental determination has shown that, in any given molecule of DNA, the concentration of adenine (A) is equal to thymine (T) and the concentration of cytidine (C) is equal to guanine (G). This means that A will only base-pair with T, and C with G. According to this pattern, known as Watson-Crick base-pairing, the base-pairs composed of G and C contain three H-bonds, whereas those of A and T contain two H-bonds. This makes G-C base-pairs more stable than A-T base-pairs[1] .



A-T Base Pair **G-C Base Pair**

The antiparallel nature of the helix stems from the orientation of the individual strands. From any fixed position in the helix, one strand is oriented in the 5' ---> 3' direction and the other in the 3' ---> 5' direction. On its exterior surface, the double helix of DNA contains two deep grooves between the ribose-phosphate chains. These two grooves are of unequal size and termed the major and minor grooves. The difference in their size is due to the asymmetry of the deoxyribose rings and the structurally distinct nature of the upper surface of a base-pair relative to the bottom surface.

The double helix of DNA has been shown to exist in several different forms, depending upon sequence content and ionic conditions of crystal preparation. The B-form of DNA prevails under physiological conditions of low ionic strength and a high degree of hydration. Regions of the helix that are rich in pCpG dinucleotides can exist in a novel left-handed helical conformation termed Z-DNA. This conformation results from a 180 degree change in the orientation of the bases relative to that of the more common A- and B-DNA[1] .

HCV is one of the most frequent causes of liver disease with an estimated 200 million persons in the world being chronically infected[2,3] leading to acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma[4]. HCV is considered the most common aetiology of chronic liver disease in Egypt, where prevalence of anti-HCV is ~ 10-fold greater than in the United States and Europe[5,6] . In Egypt anti -HCV prevalence is estimated at 12% up to 18% in the general population and exceeds 30% among adults older than 30 years[7,8] . Its transmission is clearly associated with the parenteral route; however,

epidemiological surveys demonstrate that body fluids other than blood, including saliva, might be potential sources of HCV infection[9] . The detection of HCV in saliva was studied by many authors[10-14] . Although the detection of anti-HCV is carried out on serum, the collection of oral fluid is non-invasive, safe, simple and cheap[15]. The aim of this study was to determine the prevalence of HCV infection among high risk Egyptian children and to evaluate the detection of anti-HCV and/or HCV RNA in saliva against their detection in serum.

Patients and Methods

This study was carried out on 200 children (92 males and 108 females) whose ages ranged between 4-12 years (mean of 7.96 ± 2.6 years). They were attendants of Haematology Clinic at Abu El-Reish Pediatric Hospital, Cairo University, for receiving frequent blood transfusions. Paired serum/saliva samples were collected.

Sampling of serum and saliva samples

Serum samples, processed within 1 hour of collection by venous puncture, were stored at -80^{0}C refrigerator till the assay. Each participant provided oral fluid specimen by Saliva Sampler (manufactured for Saliva Diagnostic Systems, Inc., Medford, NY 11763) by M.M.L. Diagnostic Packaging, Inc. Troutdale, OR 97060). Saliva samples were collected by "Saliva Sampler" which was intended for the collection and the transport of saliva samples for medical and research purposes. According to the manufacturer's recommendations, saliva samples were collected and stored at -80^{0}C refrigerator till the assay. All studied children were subjected to thorough history taking, complete clinical examination and laboratory assessments including:

Liver function tests

AST, ALT, alkaline phosphatase, total proteins and albumin. Kits used were Bio Merieux, France. Total and direct bilirubin was measured by Boehringer Manheim kit, Germany.

Markers for HCV

Determination of anti-*HCV* by ELISA technique (using kit supplied by Murex; version IV) was performed for serum samples of all studied children and for saliva samples of anti-HCV seropositive children.

Determination of HCV RNA

Serum samples of anti-HCV seropositive children were tested for HCV RNA by a 'direct' home made RT-PCR method described by Abdel-Hamid *et al.*[16] . Negative cases were repeated after HCV RNA extraction by using total RNA system of Promega isolation extraction kit (USA). Saliva samples of HCV RNA seropositive children (n=79) were assessed for the presence of HCV RNA by the same method described for serum samples after HCV RNA extraction from the start.

Sensitivity of the technique was determined by studying the products of PCR at serial serum dilutions. Serum of an HCV infected patient with viral load of 2.5 x 10^6 copies/ml and serum of a healthy HCV negative volunteer were used. At first 100 µl of patient's serum were added to 150 µl serum of a healthy HCV negative volunteer to obtain $1x10^6$ copies /ml dilution, then 20 µl out of this dilution were added to 180 µl of HCV negative serum giving rise to $1x10^5$ copies/ml and so on resulting in $1x10^4$ copies /ml and $1X10^3$ copies/ml. Then equal volumes (of the diluted and negative samples) resulted in a dilution of 500 copies/ ml which was repeated giving rise to 250 copies/ml. Out of this dilution 100 µl were added to 150 µl of HCV negative serum resulting in 100 copies/ml then, 100 µl: 100 µl of negative sample resulted in dilution of 50 copies/ml. Dilutions of saliva of the same patient was also performed after HCV RNA extraction. The detection limit of the technique was 50 copies/ml as shown in the following figure.

Dilution of a high viraemia case (2.5 x 10^6 copies/ml. Positive bands from 1 to 7 which have dilutions of 10^5, 10^4, 10^3, 500, 250, 100 and 50 copies/ml, respectively.

Statistical analysis

The results were expressed as means and standard deviation (±SD). The student's paired t test was used for pair wise comparison between values. $p<0.05$ was considered significant.

Results

Among 200 studied children 134 (67%) children were anti-HCV seropositive, out of them 59% (79/134) children were also HCV RNA seropositive without a significant difference between anti-HCV seropositives and anti-HCV seronegatives regarding to age or gender. The prevalence of HCV infection among the studied children was 39.5% (79/200).

Regarding to the results of liver function tests, no significant differences ($p>0.05$) were observed between HCV RNA seronegative and seropositive children in the activities of AST (55.0 ± 36.3 U/L vs. 61.5 ± 44.0 U/L), ALT (18.2 ± 10.4 U/L vs. 20.4 ± 14.2 U/L), alkaline phosphatase (134.3 ± 64.4 U/L vs. 111.7 ± 43.0 U/L) and in the levels of albumin (5.2 ± 0.8 g/dl vs. 3.6 ± 0.6 g/dl), total proteins (7.2 ± 1.2 g/dl vs. 7.4 ± 1.5 g/dl), total bilirubin (1.44±1.11 mg/ dl vs. 1.48 ± 0.78 mg/dl), direct bilirubin (0.35 ± 0.17 mg/dl vs. 0.45 ± 0.27 mg/dl).

Salivary anti-HCV was tested for 134 anti-HCV seropositive children and results revealed that 68 (50.7%) children had anti-HCV in saliva as well. Estimation of HCV RNA in saliva was performed to only 79 HCV RNA seropositive children and results revealed that 31/79 (39.2%) children had HCV RNA in saliva as well. The frequency of serum HCV RNA positive samples of anti-HCV positive children was 59%. The frequency of HCV RNA in saliva samples among serum HCV RNA positive children was 39.2%. These are represented in the following table.

	Negative		**Positive**	
	No.	%	No.	%
Anti-HCV detection in serum by ELISA (No=200)	66	33	134	67
Anti-HCV detection in saliva by ELISA (No=134)	66	49.3	68	50.7
Serum HCV RNA detection (No=134)	55	41	79	59
Salivary HCV RNA detection (No=79)	48	60.8	31	39.2

Frequency of anti-HCV and HCV RNA in serum and saliva of the studied children.

Discussion

HCV infection is the most common cause of chronic liver disease in Egypt[17,18] . Children transfused repeatedly with blood or blood products are at high risk for HCV infection[8,19,20] . The search for HCV in body fluids other than blood is important[20] . The usage of oral fluid for detection of anti-HCV could be a useful tool for epidemiological purposes, especially when blood collection is difficult (small children, injecting drug users, haemophiliacs) or for field collection of samples in developing countries. Although reliable tests to detect antibodies in serum are used widely, assays for testing oral fluid are not available commercially at present [15] .

This study was conducted to assess the prevalence of HCV infection among high risk group of Egyptian children as well as to evaluate the detection of anti-HCV and HCV RNA in saliva against their detection in serum among HCV positive children.

Among 200 studied children 134 (67%) were anti-HCV seropositive, out of them 79 (59%) children had HCV RNA in their sera. Anti-HCV seropositivity reflects prior HCV infection but not necessarily a current liver disease[21] . It is in agreement with Sehgal *et al.*,[22] who stated that infection risk for HCV increased significantly with infusion with large amounts of infected blood or repeated percutaneous exposure to infected blood. Our results are also in accordance with El-Raziky *et al.*,[23] as they studied 105 Egyptian children who were anti-HCV seropositives to determine the outcome of HCV infection. History of blood transfusion was found in 77% of 105 children and HCV RNA was detected in 58.1% of 74 children among whom 54.1% had persistently elevated aminotransferase (ALT) levels. They concluded that blood transfusion remains a major risk for HCV transmission among Egyptian children. They added that HCV infection is not always benign in childhood period and ALT levels remained elevated in half of the children and histological abnormalities were detected in three quarters of HCV RNA positive cases.

Regarding to the prevalence of HCV infection, our data showed that 67% of 200 children were exposed to HCV infection and 39.5% of 200 children (59% of 134 anti-HCV seropositives) acquired HCV infection with HCV viraemia. In 1993, Khalifa *et al.*[24] detected a high prevalence of HCV infection among multiply transfused Egyptian children; it was 83% in patients with haemophilia, 73% with thalathaemia, and 60% with hypoplastic anemia. Most of our children were suffering from thalathaemia. Therefore, this prevalence reduction in our study may be because of the exclusion of high-risk donors and testing of donated blood for anti-HCV which have reduced the estimated frequency of transmitted HCV infection. A study from UK investigated the feasibility of surveying HCV infection among healthy children using an oral fluid specimen. It was found that 2.8% were anti-HCV positive. It was concluded that oral fluid collection is the more acceptable to children[25]. Their children were healthy children at low risk while ours were at high risk. No significant differences were observed between HCV seropositives and seronegatives in accordance to age, gender or liver function tests. This is in accordance with Goncalves *et al.* and Hermida *et al.*[26, 27] .

To evaluate the detection of anti-HCV and HCV RNA in saliva against their detection in serum, our results of serum samples revealed that 134/200 (67%) and 79/134 (59%) were anti-HCV and HCV RNA seropositive, respectively. Saliva samples of HCV infected children (n=79) showed that 53/79 (67.1%) and 31/79 (39.2%) were anti-HCV and HCV RNA positive, respectively. Previous studies showed controversial results. Some showed lower values while others showed higher ones but most of them agreed that HCV RNA can be detected in saliva of HCV positive patients. Our results of HCV RNA detection in sera (59%) and in saliva (39.2%) were higher in saliva samples than those observed by Goncalves and his coworkers[26] who found that 82% of 39 HCV positive patients (chronic hepatitis and cirrhosis) had HCV RNA in serum samples, while 8/39 (20.5%) had HCV RNA in saliva samples. They observed total concordance between HCV genotype detection in serum and saliva samples from the same patient. Maticic *et al.*[28] reported that frequencies of HCV RNA in sera and saliva samples of 48 chronic hepatitis patients were 100% and 35%, respectively. In 1995, Sugimura[29] and his coworkers found that HCV RNA was detected in 44% and 36% in sera and saliva samples, respectively of 76 patients with chronic hepatitis. Although HCV RNA detection in sera of 15 chronic hepatitis patients was 100%, salivary HCV RNA frequency was 26.6% as reported by Becheur *et al.*[30]. In Italy, Mastromatteo *et al.*[31] found HCV RNA in 44% of their examined saliva samples and 39% of these were found serum HCV RNA negative. In 1999, El -Medany *et al.*[32] found that the frequencies of anti-HCV positivity in serum and saliva samples were 82.2% and 37% of 45 chronic liver disease patients vs. 72.2% and 13.6% of 44 non liver disease patients. Among 59 patients coinfected with HCV and human immunodeficiency virus (HIV), HCV RNA was detected in 76.3% and in 37% in sera and saliva, respectively (Rey *et al.*[33]. Another study reported that HCV RNA was detected in 75% and 0% of sera and saliva samples, respectively of 102 HCV- HIV-coinfected patients [34] . In Japan, Suzuki *et al.*[20] quantitatively determined HCV RNA in 31% of saliva

samples and in 85% of gingival crevicular fluid (GCF) of 26 HCV positive patients. They observed that 46% of GCF samples had viral titers similar to or greater than those observed in the sera. So, HCV RNA detection in GCF may be a promising mean for HCV diagnosis by using body fluids.

Detection of HCV RNA in saliva of 39.2% of our children is lower than those reported by others.

In Spain, Eirea *et al*.[11] reported that the prevalence of HCV in saliva of 75 HCV patients and 75 HCV-HIV-coinfected patients was 52% and 65%, respectively. They concluded that there was a trend toward a higher HCV RNA prevalence in the saliva of HCV-HIV- coinfected patients. In Italy, Fabris *et al.*[35] reported that HCV RNA was detected in sera of 39/45 (86.6%) and in saliva of 22/39 (56.3%) of 45 patients with chronic hepatitis. They added that in all of the latter, the presence of HCV RNA was restricted to the cell fraction of saliva. We used whole saliva samples of our children. In 2002, Hermida *et al*.[27] reported that salivary HCV RNA prevalence was 52.4% of 61 HCV RNA seropositive patients. They applied a highly sensitive technique with a detection limit of 10 HCV RNA copies/ml, while our detection limit was 50 HCV RNA copies/ml. Diz Dioz *et al.*[10] demonstrated HCV RNA in saliva samples of 26/ 44 (59%) of patients with chronic hepatitis and Lins *et al*.[9] reported that HCV RNA was detected in 100% of saliva samples of chronic hepatitis patients with viraemia. The lower frequency of HCV RNA in saliva samples observed in our study may be attributed to a fact that their studies were generally based on patients diagnosed with chronic HCV infection while our study based on children at high risk "receiving frequent blood transfusions". Also, it may be due to a quantity of HCV RNA in saliva samples below the level of PCR sensitivity. Various studies have compared viral load in sera of HCV infected patients with the presence of HCV RNA in saliva and found a positive correlation[11,14,27,35,36]. However, some studies did not find this correlation[9,33]. On the other hand, other investigators reported that they were able to detect HCV in saliva samples from patients but could not detect HCV in their blood[20,31,37-39]. A possible explanation for such findings is the fact that HCV can replicate in oral epithelial cells in patients without HCV in blood[39,40]. In addition, in our study sera of anti-HCV seropositive children were analyzed for detection of HCV RNA in serum and positive cases only (n=79) were further tested for the presence of HCV RNA in saliva. Therefore, some number of our children may be misdiagnosed owing to the presence of infected serum-negative but saliva-positive subjects for the presence of the virus and we did not evaluate them. The variations in HCV RNA frequencies in saliva samples in different studies may be also due to the sampling methods used. In one study, oropharyngeal washes were used as the sampling method giving 20% HCV RNA positivity[41] , whereas in another publication, oral sponges (Salivette and Omnisal) were used, but the authors were unable to detect HCV RNA in the saliva of HCV and HIV coinfected patients[34]. Cell-free saliva samples from anti-HCV positive patients resulted in 20.5% positivity[26]. We used whole saliva samples.

Finally, our detection of HCV RNA in saliva of HCV infected children suggests a role of oral fluids in intrafamilial spread of HCV infection. A large number of hepatitis C carriers were reported in whom no route of infection can be identified[27,42]. This suggests a possible role of biological fluids in intrafamilial spread of HCV[31]. In Egypt, Mohamed *et al.*[8] studied the incidence of HCV infection and associated risk factors. They found that 67% of seroconverters were younger than 20 years of age and they suggested that transmission of HCV is occurring between family members by unknown routes. They suspected a role of saliva in intrafamilial transmission. Saliva can contain a range of infectious agents and despite of several antimicrobial mechanisms, transmission of these can occur[19]. Experimental infections by HCV contaminated saliva inoculated in non-human primates[43] and HCV transmissions following human bites [44,45] have been reported.

These findings obtained provide important implications for medical personnel regarding HCV transmission in health care settings as well as for HCV epidemiology, as the origin of the HCV viral infection remains unclear in up to 40% of cases [20] .

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امكانيـة تواجـد الحـامض النـووى لفيـروس سـى فـى لعـاب الأطفـال المصريين الذين يعالجون بنقل الدم المتكرر

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تنتشر الاصابة بمرض الالتهاب الكبدى الفيروسى ج فى جميع أنحاء العالم ومن بينها مصر ويعد الدم هو المصدر الرئيسى لتشخيصه . وقد نشرفى كثير من النشرات العلمية وجود ذلك الفيروس فى سوائل الجسم المختلفة ومن بينها اللعاب الذى يمثل وسيلة أسهل من الدم للتداول للتشخيص وخصوصاً فى حديثى الولادة و الأطفال. لذلك أجريت هذه الدراسة على مائتى طفل من المترددين على وحدة أمراض الدم بقسم الأطفال بكلية الطب بجامعة القاهرة لنقل كميات معوضة من الدم لدراسة نسبة الإصابة بعدوى الفيروس الكبدى ج وكذلك الأجسام المضادة له بلعابهم ومقارنتها بوجودها فى عينات الدم الخاصة بهم. وقد أخذ التاريخ المرضى ، وتم الفحص الإكلينيكى لكل منهم. وقد تم قياس وظائف الكبد المختلفة مثل" : ألنين امينوترانسفيراز-أسبارتيت أمينوترانسفيريز -الفوسفات القلوى- ألبومين -بروتينات كلية -صفراء كلية ومباشرة ."وقد تم عمل دلالات الأجسام المضادة للفيروس ج لجميع الأطفال فوجدت إيجابية فى ١٣٤ طفل من الأطفال (بنسبة ٦٧ ٪) . قمنا بالبحث عن الحامض النووى " RNA" للفيروس بالتفاعل التسلسلى التراكمى له فى الدم لهؤلاء الأطفال فكان إيجابياً فى ٧٩ طفلاً (بنسبة ٥٩ ٪ ممن تعرضوا للإصابة بالفيروس وبنسبة ٥و٣٩ ٪ من مجموع الأطفال كلهم الذين أجريت عليهم الدراسة).

وهم يمثلون الأطفال الذين قد أصيبوا بالفعل بالمرض. فبحثنا فى عينات لعابهم عن وجود الأجسام المضادة للفيروس ج وكذلك عن الحامض النووى له وكانت النتيجة إيجابية فى ٧٩/٣٥ طفلا (بنسبة ١و٦٧٪) بالنسبة للاجسام المضادة و٧٩/ ٣١ طفلا بنسبة ٣٩و٢ ٪ بالنسبة للحامض النووى " RNA" للفيروس الكبدى ج. ومن هنا يتبين لنا أن ٦٧٪ من أطفال هذه الدراسة قد تعرضوا بالفعل للاصابة بالفيروس وقد أصيب منهم بالمرض فعلا ٥و٣٩ ٪ من كل الاطفال. ومن هنا يتبين أن هذه النسبة تعد مرتفعة بالنسبة لدول العالم المتقدم بالرغم من أنها أقل مما سبق فى دراسات أخرى. ومن هذه النتائج فإن ظهور الفيروس نفسه فى لعاب الطفال المصابة بالمرض يشير لدور ما للعاب فى إنتشار العدوى بالفيروس الكبدى ج وكذلك فهى مؤشر يمكن الاستفاده منه فى التشخيص بدلَ من الدم فى بعض الحالات.