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EVALUATION OF ANTI-HEPATITIS A VIRUS IMMUNOGLOBULIN M IN URINE SAMPLES FOR RAPID DIAGNOSIS OF HEPATITIS A IN CHILDREN

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Abstract
Children are the most frequently infected group by Hepatitis A virus (HAV). Viral hepatitis infections are frequently observed in preschool age, among schoolchildren and young adults, and within closed institutions. Frequently, it is difficult to collect blood samples, especially from infants, children, and individuals to whom access is limited. Urine samples are easier to collect, the collection method is not invasive, and collection does not require qualified staff. In addition, urine samples can be tested without previous concentration or treatments by using a class-specific antibody capture assay. The main goal of this study was to test the feasibility of using urine as a specimen for detection of anti-HAV antibodies IgM for diagnosing HAV infections. A correlation of 90.78% between the test results of urine and serum samples was obtained. The levels of anti-HAV immunoglobulin M (IgM) antibodies capture enzyme-linked immunosorbent assays for hepatitis A were performed on paired serum and urine specimens collected from hepatitis A patients (n = 100) and healthy individuals (n = 50). Hepatitis A patients seropositive for anti-HAV IgM showed 95.85% uropositivity. No false-positive reactions were observed in control groups. The uropositivity of anti-HAV IgM persisted during the convalescent phase of the disease. Using seroreactivity as a "gold standard," the sensitivity and specificity for anti-HAV IgM, tests with urine as a specimen were found to be 95.65 and 76.47%. Urine appears to be comparable to serum for diagnosis of recent infection with hepatitis A.
**Introduction**

The immune response against HAV consists initially of immunoglobulin M (IgM) antibodies, which is detectable at the time of jaundice appears. It is therefore important in laboratory diagnosis of HAV. The appearance of IgM is followed 1-3 weeks later by the production of Immunoglobulin G (IgG) antibodies, which provides lifelong protection\(^1\).

Blood samples are of prime importance in biochemical testing and in seroimmunological diagnosis. Collection of blood specimens, however, is cumbersome on account of the need for sterile equipment and trained staff. In many developing countries, the use of disposable syringes, needles, and gloves is not regularly practiced, rendering the subjects at risk for infections. A slippery vein or improper judgment of the location of a vein gives rise to untoward reactions. To circumvent the need for blood samples, the potential of alternative body fluids such as saliva and urine for detection of immunoglobulins against various microbial agents has been investigated\(^2,3\).

Urine is a body fluid with low concentrations of immunoglobulins. It has been postulated that large macromolecules such as immunoglobulin M (IgM) antibodies cannot pass through the glomerular filter under normal conditions. However, monomeric IgM proteins (67,000 kDa) have been detected in postrenal sources and not in the glomerular filter\(^4,5\). The utility of urine for diagnostic testing has been reported for many viral infectious diseases\(^6,7\). Particularly for hepatitis A, Joshi et al. have found that urine appears to be comparable to serum as a clinical specimen for the diagnosis of recent and past infections\(^1\).

Among the assays employed for detection of salivary or urinary antibodies against infectious agents, antibody class capture assays were preferred to conventional assays\(^8,9\). The capture assays have been reported to be dependable due to their abilities to capture specific immunoglobulin even at low levels and to establish specificity in the initial stage of the assay. Immunoglobulin M (IgM) and IgG capture radioimmunosorbent assays have been demonstrated to detect urinary and salivary anti-hepatitis A virus (anti-HAV) antibodies\(^4\). An IgG capture enzyme-linked immunosorbent assay (ELISA) has been attempted for detection of antibodies to respiratory syncytial and influenza A/Taiwan (H1N1) viruses in urine\(^10\). However, satisfactory use of IgG capture ELISA for detection of salivary and urinary antibodies against human immunodeficiency virus (HIV) types 1 and 2 has been described\(^8,11\). This assay appeared to be a promising alternative to conventional tests for use as a new epidemiological tool for surveillance purposes\(^12\).

**Materials and Methods**

Paired serum and urine samples were collected from 100 jaundiced children "patients group" and 50 apparently healthy children "control group", no history of recent illness was reported for control group. All cases were selected from Pediatric Outpatient Clinic, Al-noor specialist Hospital from Feb. 2010 to Octob. 2010. The patients were clinically examined for characteristic symptoms and signs and elevated serum liver enzymes "ALT & AST " levels . Patients group included 65 males and 35 females, with age range 5 to 11 years, control group included 24 males and 26 females with similar age range. Prior to sample collection, informed consent was sought from their parents. All serum and urine specimens were stored in aliquots at -20 and -70°C, respectively until processed for ELISA.

A class-specific capture ELISA was used to detect anti-HAV IgM antibodies in both serum and urine samples. The cutoff value was determined by adding 1/10 of the mean optical density (OD) from duplicate determinations with a known positive control sample to the mean OD from triplicate determinations with a known negative control sample\(^13\). Specimens with absorbance values less than and greater than the cutoff value were considered negative and positive, respectively\(^14\).

The highest 10 urine samples concentrations were collected and tested at 1.2 and 3 months after the onset of the disease to compare it with blood samples at the same intervals.
Twenty positive urine samples were used to evaluate the level of anti-HAV IgM antibodies after storage under refrigeration (4°C) at 48 to 72 h after collection.

Another 20 positive urine samples were used to evaluate the stability of anti-HAV IgM antibodies stored at -70°C for 6 months.

Statistical analysis was performed by using the Statistica statistics package. The Kolmogorov-Smirnov test, the Student t test, analysis of variance, and Fisher's exact test were used to analyze the data.

Results

A total of 150 children (100 patients and 50 control) were included in this study. The median ages of the patients were 7 years (age range, 5 to 11 years, 65% males) and for control were 6 years (with similar age range, 48% males).

Serum and urine samples were collected at the same time from all cases, and levels of serum IgM and urinary IgM against HAV were measured using ELISA. Serum HAV IgM was positive for all patients, while urinary HAV IgM was positive for only 91 (91%) cases. There was no statistically significant difference between the percent positivities of serum and urine samples (P > 0.05). Both serum and urinary HAV IgM were negative for all control cases (Tab. 1).

The sensitivity and specificity of the urine-based ELISA were 82.59 and 90.21%, respectively. A good correlation (90.27%) between the results of the urine and serum assays was obtained. The positive and negative predictive values were 91.48 and 84.52%, respectively, which is an acceptable proportion between positive and negative results and between results for infected and healthy individuals.

Twenty urine samples were used to evaluate the stability of anti-HAV IgM antibodies stored at -70°C for 6 months. At 6 months, 9 out of the 20 urine samples stored at -70°C tested negative, indicating loss of IgM during storage. Studies on the effects of freezing and storage conditions on the stability of urine samples have been reported.

Twenty samples were tested after storage under refrigeration (4°C), all 20 were found to be positive without any loss of anti-HAV IgM activity at 48 to 72 h after collection.

Samples were collected and tested at 1, 2, and 3 months after the onset of the disease to evaluate the kinetics of urinary anti-HAV IgM. The median value OD value of the serum samples collected at one month postinfection was 0.826, higher than that for the urine samples (0.597). Also, for the specimens collected one month later, the median OD value for the serum samples was higher value (0.294) than the median OD value for the urine samples (0.162). At 3 months postinfection, all of the serum samples tested positive, while seven (70%) out of ten urine samples tested negative. The OD median values for the urine samples collected at 1.2 and 3 months postinfection were significantly different (P < 0.01), these results indicating persistence of anti-HAV IgM for prolonged periods during the convalescent phase of the disease.

The results for the urine and serum samples from healthy children, which were used as negative controls, demonstrated that using urine samples did not decrease the specificity of the ELISA. These results provide evidence that support the use of urine samples for rapid diagnosis of hepatitis A. While the results of hepatitis cases also suggest the usefulness of urine samples in achieving a quick response for interpretation of cases.

Tab. 1: Comparison of paired serum and urine specimens for anti-HAV IgM.

<table>
<thead>
<tr>
<th></th>
<th>Serum</th>
<th></th>
<th>Urine</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. positive/ no. tested (% positive)</td>
<td>OD (mean±SD)</td>
<td>No. positive/ no. tested (% positive)</td>
<td>OD (mean±SD)</td>
</tr>
<tr>
<td>Patient group</td>
<td>100/100(100)</td>
<td>0.983±0.017</td>
<td>91/100(91)</td>
<td>0.604±0.049</td>
</tr>
<tr>
<td>Control group</td>
<td>0/50(0)</td>
<td>0.058±0.017</td>
<td>0/50(0)</td>
<td>0.043±0.014</td>
</tr>
</tbody>
</table>
Discussion

Hepatitis A virus infections are frequently observed in preschool age, among schoolchildren and young adults, and within closed institutions. Frequently, it is difficult to collect blood samples, especially from infants, children, and individuals to whom access is limited. Urine samples are easier to collect, the collection method is not invasive, and collection does not require qualified staff. In addition, urine samples can be tested without previous concentration or treatments by using a class-specific antibody capture assay.

The class-specific antibody capture ELISAs was used to detect anti-HAV IgM antibodies in both serum and urine samples. Use of urine specimens in anti-HAV IgM ELISA correctly identified 91% of anti-HAV IgM-seropositive hepatitis A patients and did not produce any false-positive reactions in control groups. The sensitivity of the test using urine may have been equivalent to that of the test used for serum. However, the urine samples classified as negative for anti-HAV IgM. Thus, the absence of IgM in 9% of urine samples indicated the absence of filtration, local synthesis, or transudation of IgM in urine or its presence below the detection limits of the IgM ELISAs employed.

Chitambar et al (1996)\textsuperscript{(14)} explain low sensitivity of their test that could have been caused by the following reasons. (i) Urine samples may not have been collected at the optimal time (the anti-HAV IgM kinetics in urine are not well known). (ii) There may have been immunoenzymatic reaction inhibitors present in the urine, due to chemicals, drugs, or toxic products which normally are excreted in urine. (iii) Differences in the amounts of liquids ingested promote fluctuation in the immunoglobulin concentrations in urine.

While Ireland, and Nicholson (1996)\textsuperscript{(15)} explain false-positive results that are probably due to the effect of the pH of the urine, bacterial contamination, or the presence of sediments in the urine of the individuals tested. All of these could interfere with or block the immunochemical reaction in the ELISA.

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Many types of microorganisms multiply rapidly in urine at room temperature. For practical purposes, urine specimens need to be processed rapidly or stored refrigerated during the time before analysis is performed\textsuperscript{(1)} In order to avoid the problem of bacterial contamination, we preferred to perform tests on fresh samples and store them in aliquots at -70°C. The stability of urine anti-HAV IgM after storage was examined. The percent positivity for anti-HAV IgM in urine specimens declined significantly to 55\% (11 of 20) (P < 0.05, chi-square test) 6 months, indicating loss of IgM during storage. Twenty samples were tested after storage under refrigeration (4°C). All samples were found to be positive without any loss of anti-HAV IgM activity at 48 to 72 h after collection.

The highest levels of anti-HAV IgM antibodies in serum are reached during the acute phase of HAV, and the antibodies often disappear 3 or 4 months after the onset of the illness. However, Raymond, S. K. (1992)\textsuperscript{(16)} demonstrated that anti-HAV IgM antibodies may persist for more than 6 months in 25% of patients. Our results have shown that the level of anti-HAV IgM antibodies seems to decrease gradually, but it decreased faster in urine than in serum. It is remarkable that the anti-HAV IgM levels for Ten patients were higher in their urine than in their serum at the beginning of the disease. Nevertheless, this anti-HAV IgM reactivity in urine decreased significantly at 6 months postinfection (P<0.01). Further studies of this topic with more specimens may be needed.

Finally, the usefulness of urine as a specimen for diagnosis of hepatitis A, could be confirmed in large-scale epidemiological studies. If it stands the test of large sample sizes, this may find several applications in routine surveillance, epidemiological investigations, and hepatitis A vaccination programs.

References


10- Ireland et al.,


