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HIV ANTIBODY DETECTION USING AN ULTRASOUND PARTICLE AGGLUTINATION METHOD

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ABSTRACT

HIV infection is one of the major problems in public health. In general, HIV antibody testing of clinical samples begins with the screening of test samples by an assay that has a high sensitivity, usually an ELISA (enzyme-linked immunosorbent assay). These tests, however, are time-consuming and require expensive equipment. Therefore, simpler rapid assays for HIV antibody detection have emerged over the last decade as alternatives to the ELISA. It is in this context that we developed a new, simple and sensitive ultrasound particle agglutination method (UPA) for the detection of HIV antibody in clinical samples.

In the present work, we developed several acoustical cylindrical resonator cells, examined and optimized their performance, developed an electronic unit for controlling the ultrasonic system, developed data analysis algorithms with corresponding software, and built a functional proof-of-concept UPA system. For efficient conducting the UPA method, the regime of ultrasonic exposure of samples was optimized.

Using the developed instrumentation, we tested more than 50 clinical samples, and showed that the UPA does not produce false positive results, while allowing an increase in the sensitivity of a conventional particle agglutination assay (Capillus HIV test, Trinity Biotech, Ireland) by 40-80-fold; it also reduces the time of analysis by 5-fold, and saves on costly reagents by 30-times.

INTRODUCTION

The first cases of AIDS in the United States were reported in June of 1981. Since then, more than 1.5 million people in the U.S. have been infected with HIV, including more than 500,000 who have already died [1]. HIV testing is important for both prevention and treatment efforts. At present, classical HIV tests such as ELISA and Western blot (WB) are supplemented by multiple new technologies that offer advantages for various testing situations [www.fda.gov/cber/products/testkits.]. New technologies include HIV detection in various human liquids such as saliva, urine and whole blood [2-4], simultaneous detection of both HIV antigen and antibody [5], molecular tests for HIV genotyping, viral load tests, and anti-drug-resistance assays [6,7]. However, all these technologies require expensive equipment, technical expertise, and are time-consuming to perform. Therefore, a number of simple and rapid assays have emerged to meet specific demands such as HIV detection in emergency cases [8], in perinatal care to decrease transmission to the newborn [9], and in public health clinics where a significant proportion of patients never return for their initial screening test results [10,11].

Among the rapid tests that are used internationally, a particle agglutination assay offers significant cost and time savings. The principle of particle agglutination test is immunoprecipitation of antigen-coated latex beads that have bound specific HIV antibody from the sample. In its simplest format, the sample containing antibody is mixed with the antigen-coated beads on a test slide and the agglutination is observed visually to produce a qualitative “yes/no” result. However, significant limitations of the conventional particle agglutination assay include a limited sensitivity, and a subjective interpretation of test results.

At present, there is a growing need for sensitive, simple, user-friendly and cost-effective assays that can easily be implemented at clinics and laboratories for the routine diagnosis of HIV infection. These new methods are aimed at a reduction in consumption of costly biochemical reagents, minimal handling of positive samples, automatic quantification of results and miniaturization of the assay equipment.

One of the approaches toward development of sensitive and simple methods is the use of ultrasound for improving the performance of the particle agglutination assay. In particular, ultrasound has been used to shorten the time of analysis and increase the sensitivity of the conventional particle agglutination assays [12-19]. Basically, ultrasonic forces increase the probability of bead collisions needed to enable antigen-antibody cross-linking. Initially the ultrasonic technique was employed for increasing the sensitivity of a haemagglutination assay for detection of Hepatitis B virus [14]. Since then, improvements in the performance of particle agglutination immunoassays for the detection of a number of microorganisms and viruses using ultrasound have been demonstrated [12,15-19]. In the clinical context, a group of British scientists led by Dr. Coakley has been developing an ultrasound-based particle agglutination immunoassay for the clinical diagnosis of aggressive meningitis [20-23].

However, it should be noted that the technology developed by Dr. Coakley's group, even though it allows an increase in the sensitivity of the test, suffers from the same serious drawbacks that are peculiar to the conventional particle agglutination technique. Indeed, a common "side effect" of any agglutination assay is an appearance of non-specific agglutination [24]. Ultrasound leads to an increased probability of both specific and non-specific cross-linking [12, 25]. In ultrasound "enhanced" assays the attempts to decrease non-specific agglutination are accomplished by using silica beads instead of latex [25] and/or by incorporating a physical step (manual stirring, shearing forces etc.) for disruption of non-specific aggregates. Since most agglutination tests are based on the use of latex beads, and physical steps are often inefficient, these approaches do not completely solve the problem of non-specific agglutination.

It is in this context that we developed a quantitative, fast and highly sensitive ultrasound particle agglutination (UPA) assay for the detection of HIV antibody, which is free from the above drawbacks of the conventional and previously developed ultrasonic-based particle agglutination methods. In particular, we developed a new approach to disintegrate non-specific aggregates by using a multifrequency method of manipulation of particles in a cylindrical ultrasonic resonator. In addition, the quantitative assessment of immunochemically bound aggregates using simple optical means has been developed. In the present article, we show that the UPA method does not produce false positive results, allows an increase in the sensitivity of conventional particle agglutination assays by 30-80-fold, reduces the time of analysis by 5-fold, and save on costly reagents by 30 times.

MATERIALS AND METHODS

Conventional particle agglutination method. The Capillus HIV-1/HIV-2 test kit from Trinity Biotech (cat # 6048G) for the detection of antibodies to HIV-1/HIV-2 was used to develop the UPA. The conventional agglutination procedure was performed according to the manufacturer's instructions. Briefly, positive control serum (lot # N 25818) was diluted 1:2, 1:4, 1:8 , 1:16 and 1:32 using normal human serum (Sigma, cat # H4522). The HIV antigen-latex reagent (120 μl volume) and test sample (10 μl volume) were mixed in the mixing well on the slide. The mixed reagents moved to the flow channel where the reagents flowed by capillary action towards the viewing window. The capillary flow enhances binding of antibodies to the latex and hence promotes aggregation. Following this migration and mixing, the agglutination reaction was read visually when the latex solution reached the viewing window. A smooth milky white appearance was considered a non-reactive result, whereas a change from the "milky" solution to a "sour milk" appearance was considered a positive result. The total time of analysis was 15 min.

The UPA method. The UPA laboratory instrumentation employed in the current study consisted of an electronic unit, ultrasonic cell, XDS-1A inverted microscope, SONY DCR-PC-330 digital camcorder and an original computer software program for image analysis. The ultrasonic cell was built as a cylindrical chamber using piezoceramic tubes PZT 5H with an internal diameter of 2.0 mm, a height of 1 mm and a thickness of 0.5 mm. It was glued on a Corning glass slide.

The positive control serum (HIV positive) was prepared in dilutions as described above. The reagent mixture was prepared by mixing of the latex beads and HIV positive serum in a microcentrifuge tube. Then, immediately, 4 μl of the reaction was transferred to the resonator cell of the UPA device, and exposed to an ultrasonic standing wave for 2 min at 2.8 MHz, which is within the bandwidth of the most pronounced resonance peak of the measuring cell. The ultrasound intensity was adjusted so that no measurable heating of the sample was produced. Next, the second regime of sonication, which included the sequential use of various higher harmonics of resonance oscillations were applied for 1.5 min to disintegrate nonspecific aggregates. During this second step, the image analysis software was used to quantify the

agglutination. The final results are presented as an Average Agglutination Quantity (**AAQ**) Index. Samples were tested in triplicate. An average of three measurements was calculated using Excel.

Clinical samples. Serum samples were collected patients seen at the University of Maryland Medical System, Baltimore. All samples were coded and unlinked to maintain confidentiality and were kept frozen at -20°C prior to testing.

RESULTS

Acceleration of the immunochemical agglutination reaction by ultrasound treatment

In order to study the effect of ultrasound on the speed of immunochemical agglutination reactions, we performed specific agglutination reactions with and without ultrasound treatment. For this purpose, all reagents in the Capillus HIV-1/HIV-2 test kit were brought to the room temperature. Twelve μl of positive or negative control solutions were mixed with 120 μl of latex-antigen reagent in a microcentrifuge tube. For ultrasound treatment, 4 μl sample aliquots were immediately transferred to the resonance cell of the UPA system, treated by ultrasound, placed under the microscope and sample pictures were taken using a CCD camera. Control samples (not treated with ultrasound) were kept at room temperature, 5 μl aliquots were taken at different incubation times, placed under a microscope, and the optical images were viewed. The obtained results are presented in Figure 1.

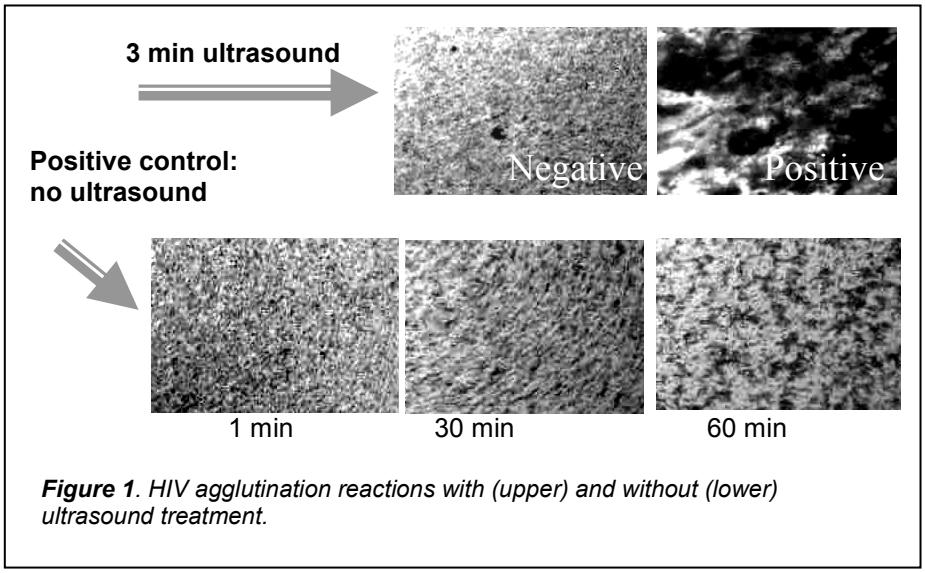

***Figure 1**. HIV agglutination reactions with (upper) and without (lower) ultrasound treatment.*

As can be seen in Figure 1 (upper row), the samples treated with ultrasound for 3 minutes show the formation of large latex particle aggregates for the positive control sample but practically, no aggregates for the negative control. The lower row of pictures obtained for the positive control sample not treated with ultrasound show that the process of formation of aggregates was not completed even after 60 min of sample incubation. Thus, ultrasonic exposure of the samples resulted in visible agglutination within several minutes whereas the particle agglutination required more than an hour with no ultrasound treatment under static conditions.

Increasing of the sensitivity of the particle agglutination test for HIV detection using ultrasound treatment

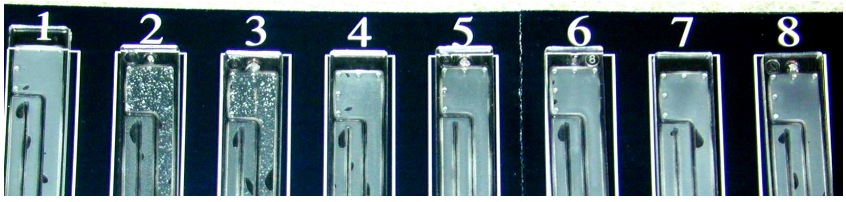

***Figure 2.** Results of testing limits of sensitivity of the Capillus HIV test. #1 - negative control; #2- undiluted positive control; #3-7 –dilutions of positive control: #3 – 1:2; #4 – 1:4; #5 –1:8; #6 – 1:16; #7 –1:32; #8 -normal human serum (Sigma cat # H4522) which was used for dilutions of positive control. Detection limit of HIV test-card is 2x diluted positive sample.*

We showed that ultrasound treatment of the samples not only decreases the time of analysis but also increases the sensitivity of HIV antibody detection as compared to the conventional agglutination method (see below). Figure 2 shows images of the

Capillus HIV-1/HIV-2 test-cards. As noted, the analytical sensitivity of Capillus HIV test is limited to the 1:2 dilution of the positive control (lot # N 25818, test card #3). Note that the detection limit is expressed as the maximal "positive control" dilution at which agglutination is detected by visual inspection of the test card.

The ultrasound particle agglutination assay was performed using the same reagents. Six µl of sample solutions were mixed with 2 µl of latex reagent. After mixing, 4 µl of the solution was transferred to the resonator cell of the UPA device and exposed to the ultrasonic field, and the image analysis software was applied to quantify the latex aggregates formed during the agglutination reaction.

The analytical sensitivity of the UPA method depends on several parameters such as resonant frequency, time of ultrasonic treatment, and the sample-latex ratio. In this work, we performed preliminary optimisation of these parameters. The most promising results obtained so far using an optimised latex-to-serum ratio and a new version of image data analysis are shown in Figure 3. For clarity, data obtained at high dilutions of the positive control is shown in the insert of the graph. As can be seen in the Figure, the assay performance for HIV detection was improved significantly. Indeed, the analytical sensitivity of the optimized UPA method was increased, as shown in the 1:64 dilution of the positive control serum. Thus, we conclude that the use of the UPA method allows one to increase the sensitvity of HIV antibody detection at least 32-fold as compared with the conventional particle agglutination assay.

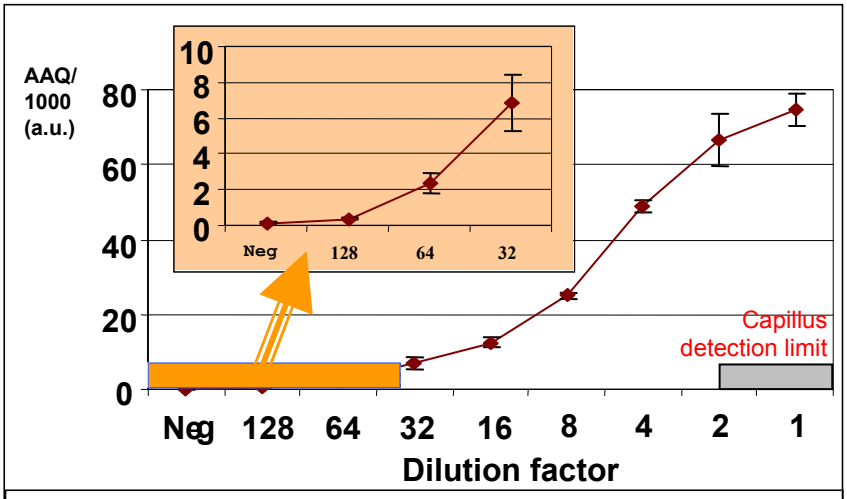

Figure 3. *Dilution curve for detection of HIV antibody obtained using the optimized assay and instrumentation conditions. Insert in the grap h is a zoom of data at high dilutions of the positive control. In the graph, the Capillus detection limit is also indicated.*

Assessment of the false positive rate of the UPA method using clinical samples

To examine the performance of UPA system for clinical samples, we used more than 50 human clinical samples: 38 human sera (set 1) initially characterized by a 2nd generation Genetic Systems HIV-1/2 test ("2nd generation ELISA") and 2 seroconversion panels (set 2) previously characterized by several 2nd and 3rd generation ELISAs, and p24 ELISA tests and Western blot tests. Here, we present the results obtained only for set 1 of clinical samples. The results obtained for 2 seroconversion panels (set 2) will be reported elsewhere.

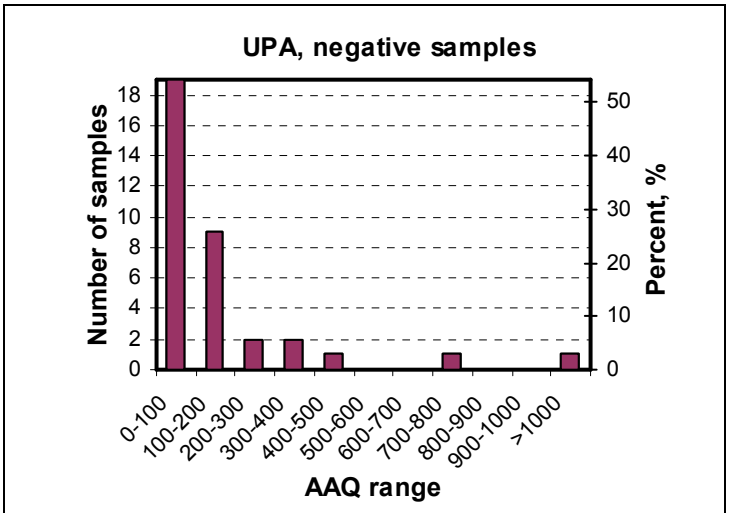

Figure 4. *The relationship between the number of 35 human sera and their AAQ value.*

The "2nd generation ELISA" test showed that 35 (out of 38) sera from set 1 were negative (free of HIV antibodies), and 3 samples were positive. We examined these samples using the 3rd generation ELISA, Genetic Systems HIV-1/HIV-2 Plus O

EIA ("3rd generation ELISA"). The "3rd generation ELISA" test showed, however, that 1 serum initially considered negative turned out to be positive. This was not surprising result because 3rd generation ELISAs are considered to be approximately 400-fold more sensitive than 2nd generation ELISAs (see Table 1 below).

In order to estimate a cut-off value of the UPA method, we used 34 true negative normal sera. The real cut-off value can be calculated as the mean (=142) + 5 SD (=124). To be conservative, we defined the cut-off value to be equal to 1000. The results obtained using the UPA method for 35 sera initially characterized by the 2nd generation ELISA as negatives are summarized in Figure 4. As can be noted in the Figure, only 1 sample had a signal higher than 1000, namely 11,295. Since this sample was also positive as determined by the "3rd generation ELISA" (see above), we drew a preliminary conclusion that the UPA method is more sensitive than the 2nd generation ELISA. Using the cutoff of 1000, none of the other samples produced a false positive reaction by the UPA method, indicating a high specificity of the UPA method.

Comparison of the analytical sensitivity of HIV antibody determination using conventional ELISAs and UPA

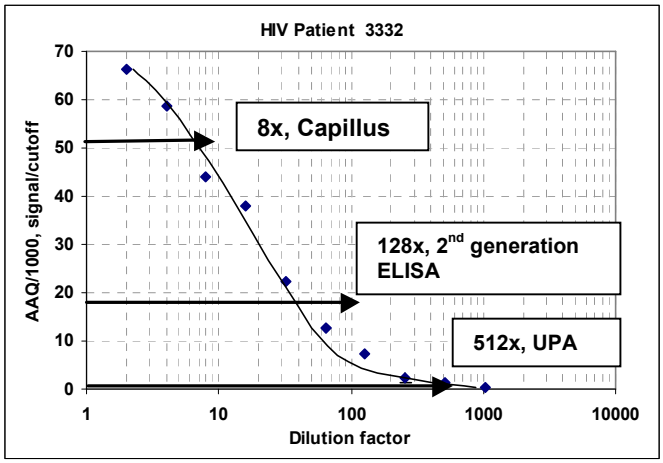

Figure 5. *Dilution curves for detection of HIV antibody in sample P3332 obtained using UPA: Raw data. In the graph, estimated detection limits for the Capillus agglutination method, 2nd generation ELISA and UPA are also indicated.*

In order to compare the analytical sensitivities of the 2nd and 3rd generation ELISAs with the UPA method, we used a positive serum (patient P3332). Figure 5 shows the dilution curve (signal/(cutoff = 1000) vs. the serum dilution) as assessed by the UPA method, and the detection limits for the Capillus test, the 2nd generation ELISA and the UPA. It should be emphasized that the detection limits for the ELISA and UPA as shown in Figure 5 were estimated as the serum dilution at which the signal/cutoff value was close to 1.

A more accurate determination of the ELISAs and UPA detection limits was obtained using regression analysis. The detection limits, expressed as dilutions of sample P3332 in negative serum at a signal/cutoff value equal to 1, and calculated using regression analysis for the 2nd generation ELISA, the 3rd generation ELISA, and the UPA method are summarized in Table 1.

Table 1. *Detection limits, expressed as dilution factors of sample P3332 for the Capillus, the 2nd and the 3rd generation ELISAs, and the UPA method.*

	Capillus	2nd generation ELISA	UPA	3rd generation ELISA
Detection limit (dilution factor)	**~ 8 - 16**	**116**	**668**	**49,300**

As we mentioned above, the Capillus test is subjectively read and qualitative, and, therefore, the detection limit for this test could be estimated only roughly. Therefore, in Table 1, the detection limit for HIV antibody detection in sample P3332, obtained using the conventional Capillus particle agglutination method is shown as a range of values rather than as one number.

As can be noted in Table 1, the analytical sensitivity of the UPA method was approximately 40-80-fold and 6-fold greater than those for the conventional particle agglutination test and 2nd generation ELISA, respectively. At the present stage of the UPA technology development, the UPA is less sensitive than the 3rd generation ELISA by approximately 75-fold. This could be explained because the 3rd generation ELISA and Capillus agglutination tests use different HIV antigens for HIV antibody capture, that may detect low

levels of different antibodies, and not necessarily because of the detection sensitivity of the UPA method [26]. Nevertheless, we believe that the sensitivity of the UPA method could be further increased upon its thorough optimisation and by using new combination of HIV antigens.

CONCLUSIONS

The UPA method, a new rapid and simple method has been developed for HIV antibody detection in human sera. The feasibility of using the UPA method for the detection of HIV antibodies in real clinical samples was demonstrated without the occurrence of false-positive results. The UPA method offers advantages to conventional HIV tests, including a short time of analysis, an increased sensitivity, quantitative results and low cost. We believe that the UPA method is a step toward expanding the use of particle agglutination technology in HIV detection.

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