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A rapid ultrasound particle agglutination method for HIV antibody detection: Comparison with conventional rapid HIV tests

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Abstract

We present the results of the feasibility and preliminary studies on analytical performance of a rapid test for detection of human immunodeficiency virus (HIV) antibodies in human serum or plasma that is an important advance in detecting HIV infection. Current methods for rapid testing of antibodies against HIV are qualitative and exhibit poor sensitivity (limit of detection). In this paper, we describe an ultrasound particle agglutination (UPA) method that leads to a significant increase of the sensitivity of conventional latex agglutination tests for HIV antibody detection in human serum or plasma. The UPA method is based on the use of: 1) a dual mode ultrasound, wherein a first single-frequency mode is used to accelerate the latex agglutination process, and then a second swept-frequency mode of sonication is used to disintegrate non-specifically bound aggregates; and 2) a numerical assessment of results of the agglutination process. The numerical assessment is carried out by optical detection and analysis of moving patterns in the resonator cell during the swept-frequency mode. The single-step UPA method is rapid and more sensitive than the three commercial rapid HIV test kits analyzed in the study: analytical sensitivity of the new UPA method was found to be 510-, 115-, and 80-fold higher than that for CapillusTM, MultispotTM and Uni-GoldTM Recombigen HIV antibody rapid test kits, respectively. The newly developed UPA method opens up additional possibilities for detection of a number of clinically significant markers in point-of-care settings.

Graphical abstract

Limit of Detection (LOD) of HIV antibody in human serum: UPA vs test kit

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Competing interests

None declared

Ethical approval

This study was approved by Essex Institutional Review Board, Inc (Lebanon, NJ), protocol No. A-092709-03. Written consent for collecting human serum samples were obtained by the organizations from which such samples were acquired. Clinical samples were coded and unlinked to maintain patient privacy.

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Keywords

HIV antibody rapid test; Immunoassay; Ultrasound; particle agglutination

1. Introduction

Acquired immunodeficiency syndrome (AIDS)¹ has become a major health concern in the last 25 years, with ~37 million people worldwide infected with HIV. In 2014 alone, there were 2 million new infections, and 1.2 million deaths (Miyagaki & Sugaya, 2011). In the absence of an effective HIV vaccine, rapid and reliable HIV testing is a critical tool for controlling the spread of HIV and AIDS, as well as for diagnosing newly-infected subjects and identifying the appropriate time for initiation of therapy. Starting antiretroviral therapy (ART) as soon after infection as possible improves quality of life and prolongs life considerably for infected patients.

Although HIV antibody tests are the most appropriate and efficient means of identifying infection, a number of other techniques are used to contribute to an accurate diagnosis, assist in monitoring the response to therapy, and to predict disease outcome. Current methods of HIV testing include immunoassays for detecting HIV-1/HIV-2 antibodies, immunoassays for detecting HIV-1 p24 antigen, and nucleic acid-based assays for detecting HIV RNA levels (Branson, et al., 2014).

Until recently, in the United States the standard of care for HIV testing consisted of initial screening with enzyme-linked immunosorbent assay (ELISA) for detection of HIV antibody (3rd generation test) and then with western blot (WB) for confirmation of HIV antibodies (Branson, 2007), (Chappel, et al., 2009), (Clark, et al., 2006). The updated Centers for Disease Control and Prevention (CDC) guidelines for HIV diagnosis recommend to use a 4th generation antibody/antigen combo screening test followed by an HIV-1/HIV-2 antibody

¹**Abbreviations:** AF, agglutination factor; LOD, limit of detection; ROC, receiver operating characteristic; UPA, ultrasound particle agglutination; USW, ultrasonic standing wave.

differentiation immunoassay (Branson, et al., 2014). These methods are labor-intensive, require expensive instrumentation and technical expertise, and sometimes take from 2 days to a week for confirmation and validation of final results (Respass, et al., 2001). Recent advances in HIV testing have resulted in a shift from the traditional approach to simpler, rapid diagnostic tests (RDTs) now approved by the U.S. Food and Drug Administration (FDA). In fact, a recent study (Waheed et al., 2013) found that several RDTs performed well enough to be considered as cost-effective alternatives to WB for the diagnosis of HIV, and can be used as confirmatory tests for HIV-1/2 differentiation after initial screening using a 4th generation ELISA under the new guidelines (Branson et al., 2014).

In the United States, the CDC and Association of Public Health Laboratories (APHL) do not currently recommend the use of RDT's for initial screening (Branson et al., 2014). The use of RDTs has, however, allowed to scale-up HIV testing services around the world, particularly in resource-limited settings. In these settings, the World Health Organization (WHO) now considers it acceptable to use 2 or more RDTs (multi-test algorithm) for initial screening to identify HIV infected individuals (Johnson et al., 2015).

The use of RDT's allows quick initial screening and identification of persons with undiagnosed HIV infection (Lyons et al., 2005). The screening helps to provide infected individuals with essential medical and preventative healthcare services (Richey et al., 2014). Of particular interest is the relation of diagnostic testing to the prevention of mother-to-child transmission (MTCT) of HIV. While rapid HIV testing is seen as an important component in the prevention of MTCT (Pai et al., 2008) (Chalermchokcharoenkit et al., 2002), the poor sensitivity of these tests have unfortunate implications on MTCT prevention programs (Black et al., 2009).

Rapid tests for HIV antibody detection mainly employ three generic formats: (1) membrane immunoconcentration (flow-through) devices, (2) immunochromatography (lateral flow strips), and (3) particle agglutination (Saville et al., 1997) (Syed Iqbal et al., 2008). Despite their attractive features such as rapidness and simplicity, rapid tests have critical disadvantages: limited analytical sensitivity (Sands et al., 2012) and, as a consequence, poor clinical performance characteristics (Villadiego et al., 2012). One area in which the performance of HIV antibody RDTs have been shown to be particularly lagging is in the identification of early (acute) HIV infection (Patel et al., 2012). Unfortunately, such performance deficiency is likely hindering efforts to eradicate HIV because transmission from those in the acute stage of infection account for approximately half of all new infections in North America (Brenner et al., 2007) and as high as ~90% of new infections in Africa (Pinkerton, 2008). Therefore, at present there is a critical need for development of sensitive, simple, user-friendly, and cost-effective assays that can be easily implemented at clinics and laboratories for the routine diagnosis of HIV infection and, in particular, capable of accurately identifying acute stage HIV infection through increased sensitivity (Bowles et al., 2008) (Jamieson et al., 2007) (Tepper et al., 2009). The new methods are aimed towards miniaturization of assay components, reduced costs, minimal handling of positive samples, and automatic quantification of results.

Among rapid tests, particle agglutination assays offer significant cost and time savings, and for this reason they are widely used in clinical practice and research settings (Dusetty et al., 2013) (Sumithra et al., 2014) (Mahat et al., 2014). One of the most popular commercial particle agglutination platforms is Serodia® (Fujirebio Inc., Japan), which can be adapted to detect antibodies against a number of viruses and microbes, including HIV (Poljak et al., 1997) (Khan et al., 2011), T-cell leukemia virus 1/2 (Pataccini et al., 2014), mycoplasma pneumoniae (Wu et al., 2009), and Hepatitis C (Ni et al., 2012), among others. Pertaining to HIV antibody detection, particle agglutination assay has an advantage to measure both IgG and IgM anti-HIV antibodies (Healey et al., 1989). The principle of particle agglutination assays for determination of HIV antibody is based on aggregation of HIV antigen-coated microbeads bound to specific HIV antibody present in the sample. Briefly, the sample is mixed with HIV antigen-coated beads on a test slide or a capillary system and agglutination is observed visually for a qualitative, binary “yes/no” result. However, conventional particle agglutination assays have drawbacks such as limited sensitivity, a relatively long time of incubation, subjectivity in evaluating the test result, and the need for large volume of reagents.

One of the approaches for circumventing the limitations of the conventional particle agglutination assay, and particularly shortening the time of analysis and increasing the assay sensitivity, is the use of ultrasound (Ellis & Sobanski, 2000) (Sobanski et al., 2002) (Wiklund & Hertz, 2006). This ultrasonic method is based on the fact that ultrasonic forces increase the probability of bead collisions required for antigen-antibody cross-linking. Initially, the ultrasonic technique was employed to increase the sensitivity of a hemagglutination assay for Hepatitis B virus detection (Grundy et al., 1989). Since then, particle agglutination immunoassays using ultrasound have been used for the detection of a number of microorganisms and viruses (Ellis & Sobanski, 2000) (Sobanski et al., 2002) (Barnes et al., 1998) (Bhaskar et al., 2002) (Gray et al., 1999) (Gualano et al., 1995) (Jenkins et al., 1997) (Sobanski et al., 2000). Though a previously-developed ultrasound-enhanced method allowed one to increase the sensitivity of these tests, this method suffers from a serious drawback peculiar to particle agglutination assays, i.e. formation of non-specific complexes, that leads to poor specificity (Wiklund & Hertz, 2006). This is mainly due to significant ‘noise’ introduced by the presence of non-specifically bound aggregates, in conjunction with subjective signal detection by the naked eye or microscope.

In order to circumvent the aforementioned drawbacks of agglutination tests using ultrasound, we developed an ultrasound particle agglutination (UPA) method, in which a novel approach to disintegrate non-specific aggregates using “multi-frequency” ultrasonic particle manipulation in a small ultrasound resonator cell is employed (Bystryak et al., 2011). In addition, in the UPA method quantitative assessment of immunochemically bound aggregates is implemented using simple optical and computational means. In our previous work (Ossina et al., 2007), we showed that the UPA method provides a fast assay with quantifiable readout for the detection of HIV antibodies. In the present paper, we describe a modified, more sensitive UPA method, and present the results of feasibility and preliminary studies on the analytical performance of the UPA method for determination of the HIV antibody. In addition, we provide results comparing the analytical sensitivity of the UPA method with those of three commercial/conventional HIV rapid tests of different formats,

namely, Capillus™ (conventional particle agglutination), Multispot™ (flow-through), and Uni-Gold™ Recombigen® (lateral flow). The results demonstrate that the UPA method is approximately 100–500 times more sensitive than these rapid tests and reduces time of analysis and reagent costs.

2. Materials and methods

2.1 HIV rapid test kits and clinical samples

Three conventional HIV rapid test kits were used: Capillus™ HIV-1/HIV-2 Rapid Test (Trinity Biotech, Ireland), Multispot™ HIV-1/HIV-2 Rapid Test (Bio-Rad, Hercules, CA), and Uni-Gold™ Recombigen® HIV-1/2 kit (Trinity Biotech, Ireland). While the prevalence of clinical use for these tests varies considerably, all are certified (as of October 15, 2015) for deployment by the USAID (United States Agency for International Development) (USAID, 2015). All reagents were used as received and test procedures were carried out under ambient conditions according to manufacturer instructions provided in package inserts.

Negative control human serum was purchased from Sigma (# H4522, St. Louis, MO). This serum was used for serial dilutions of positive control serum from various rapid test kits. Fresh negative and positive clinical serum samples were obtained from the University of Maryland Medical Center (UMMC, Baltimore, MD) and Innovative Research, Inc (Novi, MI). These samples were characterized for the presence of HIV antibodies by 3rd generation ELISA (Genetic Systems HIV 1/2 Plus O EIA). All serum samples were stored at 2–8 °C prior to use.

2.1.1 Capillus rapid test—Capillus™ HIV-1/HIV-2 rapid test (Capillus) uses polystyrene (latex) beads coated with recombinant polypeptides of envelope proteins (gp120 and gp41 epitopes) (Francis et al., 1988) (Ramalingam et al., 2002) (Thorn et al., 1987). In a recent study (Waheed, et al., 2013), the performance of the Capillus RDT led the authors to identify it as a cost-effective alternative to Western blot for HIV diagnosis. The Capillus test is carried out using a special capillary agglutination slide placed over a black interpretation card. The slide consists of a well in which the HIV antigen - latex reagent (120 µl) is initially mixed with the test sample (10 µl) and then moved to the opening of a capillary flow channel leading to a final viewing window. The latex aggregation reaction is observed visually when the solution reaches the viewing window (~ 3–7 minutes). A smooth milky white appearance is considered a non-reactive (negative) result, whereas a change to a “sour milk” appearance (white clumping) is considered a reactive (positive) result. Several dilutions (undiluted, 1/2, 1/4, and 1/8) of the positive control serum in negative control serum (Sigma) and negative control were tested.

2.1.2 Multispot™ rapid test—This test is based on the principle of immunoconcentration and is carried out using a cartridge containing a specimen pre-filter, a reaction membrane, and an absorbent pad (Zemheri et al., 2012). The reaction membrane contains four spots - one Procedural spot and three Test spots (two for HIV-1 and one for HIV-2). The two test spots for HIV-1 contain, respectively, recombinant gp41 (envelope glycoprotein) and synthetic peptide representing gp41 epitope (Wiklund & Hertz, 2006). The HIV-2 test spot

contains synthetic peptide representing envelope gp36 epitope. Typically, 30 µl sample is mixed with 300 µl Specimen Diluent in a test tube and the diluted sample is poured into the specimen pre-filter in the cartridge. After the solution is completely absorbed (~2 minutes), the pre-filter is removed and discarded. The central well of the cartridge is then filled with wash solution and allowed to absorb completely. Three drops of conjugate solution are added and allowed to react for 2 minutes. The washing step is repeated twice with the wash solution allowed to absorb completely between washes. Three drops of Development Reagent are added to the central well of the cartridge and allowed to react for 5 minutes. Finally, the cartridge is filled with stop solution, which is allowed to absorb completely before reading test results (optimally within 1 hour of testing). The membrane is examined visually for the presence of purple color on four spots. A purple color on the Procedural Control spot indicates assay validity. Purple color on HIV-1 and/or HIV-2 test spots indicate presence of HIV-1 and/or HIV-2 antibodies.

2.1.3 Uni-Gold Recombigen rapid test—The test procedure was performed using serum samples (negative, positive controls) according to manufacturer's instructions (Lefebvre et al., 1981). Briefly, one drop of sample is added to the sample port on the test device and allowed to absorb completely into the paper. Four drops of wash solution are added to the sample port. After 10 minutes of incubation (but not more than 12 minutes), test results are read visually. Two pink/red lines (one each adjacent to words "Control" and "Test") indicate a valid reactive/positive test result; a single pink/red line adjacent to the word "Control" indicates a valid negative/non-reactive test result. Absence of a line adjacent to "Control" indicates an invalid result irrespective of a line adjacent to "Test". The test spot contains rGP41, rGP36, and rGP120 epitopes (Delaney et al., 2011). It is important to note that this is only a qualitative test and the intensity of the pink/red line does not indicate the antibody levels in the sample.

2.2 UPA Method

2.2.1 General principles of the UPA method—Manipulation of aggregation of microscopic particles by ultrasonic standing wave (USW), employed in previous versions of the ultrasound-enhanced method, developed by several groups of scientists is a well-known phenomenon (Ellis & Sobanski, 2000) (Wiklund & Hertz, 2006) (Bhaskar et al., 2002) (Grundy et al., 1993). The forces responsible for redistributing particles in the liquid in accordance with the standing field pattern depend on the relative density and acoustic impedance of the particles with respect to the fluid in which they are suspended, the dimensions of the particles, and the frequency of the standing ultrasonic wave. Particles in a standing wave experience a force tending to concentrate them at radial half wavelength intervals, i.e. at positions of minimum potential energy. In this approach, the test sample containing analyte is mixed with a suspension of microparticles (beads or cells) coated with binding agent (antigen or antibody), and the mixture is placed into the capillary and exposed to an ultrasonic standing wave for times on the order of several minutes. The ultrasonic clumping process maintains the beads or cells in close proximity, thus increasing the probability of their cross-linking. The concentration of particles in the standing wave increases the rate of particle agglutination, which results in the increased sensitivity of the analyte detection. The final results are registered by the naked eye or through a microscope.

Despite significant achievements in the past, this approach suffers from the same drawbacks as other particle agglutination-based methods: subjective detection by the naked eye or microscope. Moreover, though there is an apparent increase in the extent of agglutination as compared to the conventional agglutination assays, there is also an increase in the formation of non-specific agglutinates (Ellis & Sobanski, 2000) (Thomas et al., 1999). The non-specific binding adversely affects the sensitivity and specificity of the method and is a major disadvantage of using standing wave single frequency sonication. Although manual manipulation of the sample such as tapping the capillary containing the sample to the slide to take the sample out and then sample stirring may mitigate the problem of non-specific microparticles agglutination, it introduces a new test step which may result in the test irreproducibility (Wiklund & Hertz, 2006) (Thomas et al., 1999) (Coakley et al., 2000) (Dax & O'Connell, 1999).

Our UPA method is distinct from the previous ultrasound-enhanced particle agglutination methods and is free from the aforementioned drawbacks. Major innovations implemented in our system (Bystryak et al., 2011), which allowed us to further improve the sensitivity of the ultrasound-assisted agglutination immunoassay and make it objective, include: 1) a dual mode ultrasound, wherein a first single-frequency mode is used to accelerate the latex agglutination process, and then a second swept-frequency mode of sonication is used to disintegrate non-specifically bound aggregates; and 2) a numerical assessment of results of the agglutination process. The numerical assessment is carried out by optical detection and analysis of moving patterns in the resonator cell during the swept-frequency mode.

In more detail, the first ultrasonic exposure is a single-frequency standing wave mode that causes accumulation of microparticles in the pressure antinodes of ultrasonic standing wave and results in increasing the rate of immunoagglutination reaction. As mentioned above, gathering of particles in standing wave pressure antinodes leads to the formation not only of specific aggregates caused by immunoreaction, but also non-specifically bound aggregates. In order to destroy such nonspecific aggregates, the second stage of the method, including a swept-frequency mode of ultrasound exposure, is utilized. At this stage, the ultrasound frequency is swept over a wide range. Sweeping the frequency results in a continuous change of the standing wave pattern in the tested fluid. Every time the ratio of the resonator characteristic dimension becomes close to the whole number of the acoustic half-wavelengths, a standing wave is formed in the resonator and the particles suspended in the liquid are dragged to the pressure antinodes. This, in turn, results in the continuous assembling and disassembling of particle bands, that is, in continuous jumping of the particles from one position to another. Frequency sweeping acts as a highly efficient stirrer: millions of micron-size particles move with velocities on the order of 100 mm/s relative to surrounding fluid. This results in an efficient destruction of aggregates composed of weakly-, non-specifically bound particles. After several cycles of frequency sweeping, non-specifically bound aggregates are disintegrated. The amount of the remaining aggregates comprising immunochemically strongly bound microparticles is then evaluated by optical means.

Special image processing software has been developed for analysis of dynamic patterns seen on the resonator cell during swept-frequency mode of sonication. This image analysis

software allows an accurate numerical assessment of the level of particle agglutination. To minimize the influence of the non-specific particle agglutination, the software provides recognition of moving objects, pixel clustering analysis and background noise subtraction. Depending on the nature of the test, its time takes from 1 to 5 minutes.

2.2.2 UPA instrumentation—A general view and architecture of the components of the UPA device used to obtain results in the present study are shown in Fig 1. A description of the technical specifications of the UPA hardware and software, which were developed in partnership with Artann Laboratories, Inc. (Trenton, NJ) is provided in detail in U.S. patent #7,989,177 (Bystryak et al., 2011). Briefly, the UPA device ($28 \times 8 \times 23.5$ cm) consists of a removable resonator cell (ultrasonic transducer), an electronic unit for ultrasound generation, and a data acquisition unit. An external computer controls the electronic unit, runs data acquisition, and displays results. The insert in Fig 1A shows the ultrasonic resonator cell made of a PZT-5A piezoceramic tube with an internal diameter of 2.0 mm glued to a standard $22 \times 22 \times 0.15$ mm Corning glass slide coverslip. We have shown that the cell is suitable for conducting several thousand measurements without affecting the cell's performance. A sliding tray holds and positions the coverslip in the electronic unit. The electronic unit setup allows for assessment of the ultrasonic spectrum in a preset frequency band and measurement of resonance peak parameters during a preset time. The UPA data acquisition module consists of a light emitting diode (LED), digital camcorder, and image analysis software. A graphical user interface allows for flexibly adjusting all settings of the system.

The image analysis software quantifies the degree of particle aggregation through calculation of the Agglutination factor (AF). Briefly, immediately prior to switching from the single-frequency mode to the swept-frequency mode, the particles are aggregated in a pattern characteristic of the resonant frequency. Upon transitioning to the swept-frequency mode, the particles begin to dissipate due to the continuous assembling and disassembling of particle bands. As this dissipation occurs, the UPA data acquisition system collects a series of images for analysis at a defined sampling rate of up to 50 frames per second (fps). The image acquisition software, based on the Intel Integrated Performance Primitives (IPP) library, functions in two discrete steps during the swept-frequency mode: frame-by-frame image background analysis and object motion detection and quantification of the specific agglutination process. Upon initiation of the swept frequency mode, a training sequence of images is obtained and each RGB color image within the sequence is converted to 8-bit grayscale and a restorative Wiener filter is then applied to minimize the effect of noise (Wiener, 1964). A discrete background image is created from the processed image sequence through the use of a weighted running average algorithm (weighting factor=0.5). Following formation of the background image, the second step is initiated. Within this step, the image acquisition continues at approximately 25 fps. For each frame, the background is subtracted from the captured image through pixel subtraction and the absolute value of the processed (background-subtracted) image is obtained. A sequential spatial filtering sequence, consisting of a 3×3 low pass filter, a 5×5 Gaussian filter (standard deviation=1.0), and a 5×5 low pass filter, is then applied to each processed frame/image. This process detects the motion of the particles/aggregates (relative to the background) and results in an image

consisting of these displaced particles/aggregates only. This image is then segmented using a thresholding algorithm to produce a binary image of the displaced particles/aggregates and a size thresholding algorithm is utilized to differentiate individual particles from aggregate of particles. The resulting image consists of the aggregates as areas of black pixels on a white background. The area of the aggregates, i.e. number of black pixels, in the image is quantified and this process is repeated for each frame within the image sequence. The area of the aggregates is then plotted with respect to frame number (time) within the sequence and the temporal derivative of the aggregate area is determined, which corresponds to the amount of specific agglutination and the Agglutination factor (AF) value.

2.2.3 UPA experimental procedure—The UPA experiment is carried out by loading 4 µl of 1:1 mixture of sample (serum) and latex particles coated with HIV-1/2 antigens (from Capillus rapid test kit) in the resonator cell. The resonator cell is placed in the electronic unit and a predefined algorithm is run from the computer. The parameters of ultrasonic exposure of the samples are gathered in Table 1. In this assay, the mixture is exposed initially to a standing wave of 13 MHz peak frequency for 1.5 min, followed by a frequency sweep from 12–22 MHz for 3.5 min. During the exposure period, images are displayed in real time and captured at the end of exposure. Integrated image analysis software in the computer digitizes final images and presents agglutination results as numerical values termed as Agglutination factor (AF) as described above.

3. Results

3.1 Effect of dual mode ultrasound on increase of signal-to-noise ratio in the UPA method

The example in Fig 2 illustrates dispersal of non-specifically bound latex particle aggregates by the swept-frequency mode of ultrasonic exposure. The two panels in Fig 2A show pictures of control (negative) and positive sample (1/400 dilution of the positive control serum from the Capillus test kit diluted in negative serum) after using 5 min of single-frequency ultrasonic exposure. The two panels in Fig 2B show pictures of the same samples after dual-mode ultrasonic exposure: 1.5 min single-frequency (13 MHz) followed by 3.5 min swept-frequency mode of ultrasonic exposure (12–22 MHz).

Fig 2 clearly demonstrates that most non-specifically bound aggregates are dispersed after several cycles of frequency sweeping, whereas specific aggregates remain predominantly intact. Thus, the use of dual-mode ultrasonic exposure in the ultrasonic resonator allows discrimination of specific and non-specific latex bead aggregates, resulting in increases in signal-to-noise ratio, sensitivity, and accuracy of the UPA method. It should be noted that the images depicted in Fig. 2 are captured at the end of the UPA experiment, while the Agglutination factor (AF) is obtained during the swept-frequency mode, as described above.

3.2 The analytical sensitivities of conventional rapid tests and UPA method

In order to compare the analytical performance of the UPA method versus rapid tests, specifically Capillus, Multispot and Uni-Gold, the analytical sensitivities of all four tests were determined and compared. The analytical sensitivity or limit of detection (LOD) (used interchangeably in this manuscript) is defined as the lowest concentration of an analyte that

provides a measurable test signal over assay background. It should be emphasized that the analytical sensitivity is different from clinical sensitivity that refers to the ratio (percentage) of correctly identified positive samples to the total number of positives or in our case the ability of the test to correctly identify patients with HIV infection. For the studied commercially available rapid tests, the observed analytical sensitivity is defined here as the maximal dilution of the respective positive control serum at which a visual signal was detected unaided (endpoint), i.e. milky/white clumps in Capillus test, valid purple spots in Multispot test, and valid pink/red lines in Uni-Gold test.

For the calculation of the analytical sensitivity of the UPA method, UPA experiments were conducted using the positive control solutions (sera) included in each RDT and the antigen-coated latex particles from the Capillus™ kit. These kit positive controls can be considered positive clinical samples. The use of such “convenience samples” for evaluation of diagnostic tests at the feasibility stage of development is indeed supported by recommendations from the literature (Peeling et al., 2006). Since it was assumed that the positive reference standards from each test indeed contained different concentrations of HIV antibodies, separate calibration curves (AF vs. Dilution Factor) were prepared using serial dilutions of the corresponding positive reference standard. In all cases, these positive reference standards were diluted with commercially-purchased normal serum containing no HIV antibodies (negative reference standard). Based on a non-linear regression analysis of the calibration curve, the dilution factor corresponding to the AF cut-off value was determined, as described below. The LOD, in terms of the dilution factor, of the UPA assay was determined for each positive reference standard and thus LOD was compared to the LOD for the respective RDT kit.

The Capillus rapid test is a qualitative particle agglutination test carried out using a special capillary slide with a run time of 7 minutes (Lee et al., 2012) (Windsor et al., 1997). Fig 3A shows picture of the capillary slides for 1/2, 1/4 and 1/8 dilutions of the Capillus positive control serum and negative serum. As can be seen in Fig 3A, the analytical sensitivity of Capillus HIV antibody test corresponds approximately to a 1/4 dilution of the positive control serum (Fig 3A, slide 2). It should be noted that the conventional rapid HIV tests are qualitative and highly subjective, and, therefore, their analytical sensitivity can only be approximately evaluated. In comparison, the UPA method allows for a quantifiable readout, i.e. numerical evaluation of the agglutination. Fig 3B presents a UPA calibration curve obtained using Capillus positive control serum dilutions from 1/2048 to 1/2 (labeled as dilution factor from 2048 to 2 on the graph x-axis). Compared to the negative serum (Neg) background AF, the signal (i.e. AF) at the highest dilution (1/2048) is significantly higher than background (approximately 15 times the background). It is also worth noting that the results presented in Fig 3B show that the errors for the UPA measurements at each positive control dilution do not exceed 15%, which is typical for immunoassay tests.

In order to compare the analytical sensitivities of conventional rapid tests and the UPA method, the LOD for the UPA method must be determined. For this purpose, the cut-off value, a signal corresponding to the lowest analyte concentration measurable, is defined and the corresponding LOD is calculated using regression analysis. There are several approaches for the determination of the cut-off values (Miller & Miller, 2010). During validation studies

of a clinical assay, the optimal method for determining the cut-off point is to compare the clinical sensitivity and specificity of samples by means of receiver operating characteristic (ROC) analysis to determine the best relationship between clinical sensitivity and specificity (Sharma & Jain, 2013). However, such analysis requires a large number (hundreds) of clinical samples (both positive and negative for analyte of interest) in order to accurately predict the cut-off value. Obviously, such an analysis is more suited for clinical validation studies and not the preclinical feasibility study presented in this paper.

Since the main goal of this paper is to establish the feasibility of the UPA method for determining the presence of HIV antibodies in serum or plasma, we used the following two methods to estimate the cutoff (CO) value: In the first method (method 1), cut-off value was estimated according to the formula $CO = \mu + n\sigma$, where μ is the mean of the negative serum samples, n is the whole number and σ is the standard deviation of negative serum samples (Schupbach et al., 2000). In the second method (method 2), a simplified ROC analysis was used: clinical specificities of the UPA assay were calculated for various cutoff values, and an acceptable CO was selected based on the FDA criteria.

Specifically, we evaluated a total of 52 presumably negative clinical serum samples, as confirmed by a FDA-approved HIV antibody ELISA. Each sample was tested by the UPA method as described above. Fig. 4 represents a histogram of the AF results from 52 clinical negative serum samples (bin width=50) with the x-axis representing the range of Agglutination Factor (AF) values for the samples and the y-axis representing the number of samples within each range.

As can be seen from this histogram, majority of the samples (49) exhibit AF below 500. The average AF of all 52 samples is 176 which is comparable to AF of negative control serum (Fig 3B). One sample exhibited an AF value of slightly higher than 1,000, which is much higher (> 6 times) than the average AF of all 52 samples. It is worth noting that the AF of positive serum control (Fig 3B) as well as 25 high titer HIV positive clinical samples is much higher, with AF value of $> 30 \times 10^3$, with no false negatives (preliminary clinical sensitivity is 100%).

As can be seen in Fig 4, the data distribution is skewed to the left, thus confirming the non-normality of the distribution. Since one out of 52 samples was considered a false positive and was treated as an outlier and given the skewness of the data, a log-normal probability density function (PDF) was fitted to the data for 51 samples. By utilizing the fitted PDF:

$$y = \frac{1128.22}{x} e^{-\frac{(\ln(0.0084x))^2}{1.626}}$$

the mean, median, mode, and standard deviation of the data were calculated and presented in Table 2:

As seen from Table 2, the mean AF of the 51 negative samples was derived from the PDF fit function parameters and found to be 178.46. According to the aforementioned method 1 for determination of the cut-off value, the UPA assay cut-off values as the mean of the

“negative” samples (μ) plus two, three and four times the standard deviation (σ) are 578.26, 778.16 and 978.08, respectively.

By utilizing the simplified ROC analysis (method 2), we calculated the clinical specificities for the UPA assay corresponding to the above cut-offs, and the only cut-off value which provides an acceptable clinical specificity is the $CO = \mu + 4\sigma$ rounded to 1,000. Indeed, at the cut-off value ($AF = 1,000$), the assay correctly identified 51 samples as negatives and incorrectly identifies one sample as positive (the outlier). This results in the UPA clinical specificity of 98.1%. This specificity is within the acceptable limits of current WHO requirements for use of RDTs in primary screening (Johnson et al., 2015), is in agreement with specificities of FDA-approved RDTs currently used as secondary (differentiation) tests (Branson et al., 2014), and is comparable to specificities of other conventional rapid tests (Sobanski et al., 2000) (Delaney et al., 2011). While $\mu + 2\sigma$ is a widely used expression for estimation of the cut-off value (Sharm & Jain, 2013), the $CO = \mu + 4\sigma$ rounded to 1,000 provided an acceptable UPA clinical specificity, and, therefore, was selected as the UPA cut-off value for further calculation of the UPA LOD. We realize that the current cut-off value may change with further validation studies. In the future clinical studies, a large number of clinical negative and positive samples will be evaluated and a rigorous ROC analysis will be performed to determine a validated cut-off point and corresponding LOD of the UPA method.

Thus, in the UPA method for screening of clinical samples, the sample reactivity is determined by the following rule: if ratio of AF of sample to cut-off < 1 , the sample is considered negative for HIV antibody by this test; if ratio of AF of sample to cut-off ≥ 1 , the sample is considered positive for HIV antibody by this test. Based on the regression analysis of the UPA calibration curve and a cut-off AF of 1,000, the limit of UPA detection was calculated to be 1/2040 dilution of the Capillus positive control serum. Since the analytical sensitivity of the conventional Capillus test is 1/4 diluted positive control (see above), the UPA method demonstrates a remarkable increase (510-fold) of the analytical sensitivity over the conventional Capillus particle agglutination test.

The Multispot rapid test was performed using 5 samples: 1) negative control, 2) undiluted positive control, 3) 1/2 diluted positive control, 4) 1/4 diluted positive control, and 5) human serum (Sigma), used for dilutions (data not shown). The undiluted and 1/2 diluted positive control serum exhibited valid purple spots for HIV positive reaction. Both negative serum (control and sigma serum), as well as the 1/4 diluted positive control, gave valid negative results (i.e. a purple spot only on the Procedural Control). Thus, the results show that the analytical sensitivity of Multispot rapid test is limited to approximately 1/2 dilution of the positive control serum. The analytical sensitivity of the UPA method using the Multispot positive control serum was determined in a similar manner as described above for test using the Capillus positive control serum. The signal at the highest dilution (1/256 diluted) was 6 times higher than the negative serum background. Based on the regression analysis of the calibration curve and a cut-off Agglutination Factor of 1,000, the limit of UPA detection was calculated to be a 1/230 dilution of the Multispot positive control serum. Thus, one can draw a conclusion that the use of UPA allows one to increase the analytical sensitivity of HIV antibody detection 115-fold as compared to the conventional Multispot assay.

The analytical sensitivity of the Uni-Gold test was measured using serial dilutions (undiluted, 1/2, 1/4 and 1/8 diluted) of the provided positive control serum that gave valid positive reactions only with undiluted and 1/2 diluted positive control serum. Higher dilutions (1/4 and 1/8) of the positive control serum, as well as the negative control serum, gave valid negative reactions. Thus the sensitivity of Uni-Gold test was found to be 1/2 dilution of the positive control serum. The analytical sensitivity of the UPA method using dilutions of Uni-Gold positive control serum was determined as described above. Based on the regression analysis of the calibration curve and a cut-off Agglutination Factor of 1,000, the limit of UPA detection was calculated to be 1/160 dilution of the Uni-Gold positive control serum. Thus, the UPA method exhibits an 80-fold higher analytical sensitivity than that for the Uni-Gold test.

The detection limits for UPA and three rapid HIV antibody tests obtained using corresponding positive controls are summarized in Table 3. The results presented in Table 3 show that the analytical sensitivity of the UPA method is 510-fold, 115-fold, and 80-fold higher than that of the Capillus, Multispot, and Uni-Gold tests, respectively.

4. Discussion

There are, in general, three serious drawbacks of the conventional particle agglutination methods: 1) a limited analytical sensitivity of the assay; 2) difficulty and subjectivity in interpretation of the assay results; and 3) the need for mechanical rotational motion of slides to accelerate the agglutination process.

Ultrasound is a simple and powerful option to improve analytical sensitivity of the particle agglutination method. Since 1989, ultrasonic standing wave (USW) methods have been used for enhancement of particle agglutination process (Grundy et al., 1989) (Grundy et al., 1993). The basic idea is to increase the probability of particle collisions by the use of ultrasonic forces instead of manual agitation of the sample. In previous applications, the standing-wave field was generated by reflection in the upper interface between the liquid sample and air (Grundy et al., 1989) (Jepras et al., 1989). Later, several tests based on ultrasound-enhanced cell, bacteria or latex agglutination were implemented by using slightly modified device geometries (Ellis & Sobanski, 2000) (Wiklund & Hertz, 2006) (Grundy et al., 1993). In general, the sensitivity of latex agglutination tests have been improved by USW, but the use of standing waves still can lead, in some conditions, to spurious results due to non-specific aggregation. Some efforts have been made to resolve these issues. For example, the use of silica beads instead of latex beads for the suppression of non-specific agglutination has been reported (Thomas et al., 1999). Alternatively, manual tapping of the capillary and/or stirring of the sample on a slide can be used to disrupt non-specific complexes (Ellis & Sobanski, 2000) (Sobanski et al., 2002). However, such manual manipulations result in poor reproducibility and are not suitable for working with potentially infectious biological fluids. The UPA method described here does not require manual manipulation of the sample. After reactants are placed into the resonator cell, they are incubated, exposed to the dual mode ultrasound and detected in bulk solution without performing any manual steps.

The total time required to conduct our HIV UPA test is < 7 minutes including loading reagents, exposure to ultrasound and computational analysis, which makes UPA a rapid test. In addition, there is a significant reduction of reagent and sample use. For example, the amount of latex beads is only 2 µl per UPA test in comparison with 120 µl for the Capillus test. Since the amount of sample required per UPA test is also being in the order of microliters, a finger stick whole blood, which is the sample of choice for screening rapid tests, can be used in the UPA method. Moreover, the UPA method does not require any washing or color-development steps that are used for Multispot and Uni-Gold tests.

Clearly, for optimal rapid test performance in a clinical setting, numerical readout of the signal is beneficial because it provides an objective result. Indeed, several studies have shown that there is considerable variation in the interpretation of particle agglutination results (Dax & O'Connell, 1999) (Chiu et al., 2011) (Spielberg et al., 1989). This issue also arises in the interpretation of other rapid test results and affects their performance in the clinic (i.e. clinical sensitivity and specificity). For example, a 2011 study demonstrated that interpretation of four different HIV rapid tests was inconsistent and could be improved by using photographed rapid test results allied to a 10-minute training session of operators (Chiu et al., 2011). Therefore, the UPA method providing a numerical readout of the signal has an advantage over other rapid tests using visual interpretation of the results.

A direct comparison of the analytical sensitivity of the UPA method with those of the conventional rapid tests may seem ambiguous due to the nature of endpoint measurement (numerical readout vs. visual interpretation). We realize that instrumentation-based rapid HIV antibody assays with numerical readout have recently emerged on the market (e.g. Bio-Rad Geenius), and may provide higher analytical sensitivity than assays with the visual signal detection. The preliminary data reported for Geenius test shows, however, that this is not the case, and its seroconversion sensitivity (linked to analytical sensitivity) is comparable with that for Multispot (Malloch et al., 2013). Since the UPA method is a rapid test, the comparison of its analytical performance with those for other rapid tests irrespective of the detection system they use makes sense. It is worth noting that Multispot and Alere Determine HIV-1/2 antibody tests are among the most widely used and most sensitive conventional rapid HIV antibody tests available on the market (Masciotra et al., 2013). For this reason, the Multispot assay, despite that it is a confirmatory (supplemental) test, among the others, was selected for comparison with the UPA method.

Agglutination can be measured by using spectrophotometers, nephelometers, scanning laser microscopy, magnetic moment analysis, photon correlation spectroscopy, fluorescence analysis, etc. (Delanghe et al., 1990) (Medcalf et al., 1990). Although instrumental methods have been applied to a wide variety of commercial assays, they are not generally used for rapid tests because of the expense of instrumentation. Remarkable progress in electronics and optical imaging has been observed in recent years, which has allowed us to develop a small and inexpensive UPA device that combines ultrasonic exposure and optical imaging of agglutination. Notably, the UPA device can be further simplified. For example, the present version of the UPA device includes a laptop computer for controlling the system and processing the data, but the system could equally well be implemented using a smart phone or similarly compact and inexpensive device. For resource-limited settings we project that an

integrated single board computer with touch screen monitor would fully satisfy the requirements of the UPA system. The system will also include a rechargeable battery pack (four NiMH battery type C), which will provide approximately 5 hours of continuous operation. Another feature of the UPA method is that it allows one to use disposable measuring (ultrasonic) cell which is now being developed. Such a disposable cell will also be equipped with a membrane for separation of red blood cells and plasma (Wang et al., 2014), thus allowing to conduct the test using whole blood. Our goal is to develop not only a very sensitive but also inexpensive device. Our estimates show that we are quite close to that goal: costs of all major components of the device will hardly exceed \$300.

It should be noted that in our UPA experiments the Capillus latex beads were used for evaluation of positive serums from Capillus, Multispot and Uni-Gold rapid tests. These kits use different cocktails of antigen epitopes as mentioned earlier. We believe that the analytical sensitivity of the UPA method can be further improved by optimizing the combination of specific HIV antigens on the latex beads.

The capability of the test to detect IgM anti-HIV antibody is very important because this type of antibody appears earlier than IgG anti-HIV antibody, and thus HIV infection could be detected earlier. Unlike 2nd generation ELISA tests that can detect IgG antibody only (Pandori et al., 2009), tests that can detect both IgM and IgG anti-HIV antibody relate to the third generation ELISA tests and are based on the use of HIV antigens which “sandwich” anti-HIV antibody. Although it has not been shown directly yet, the UPA method is most likely capable of detecting both IgM and IgG anti-HIV antibodies, which makes the UPA test a third generation assay. This confidence is based on the fact that a particle agglutination assay similar to Capillus has been shown to detect IgM anti-HIV antibody (Healey et al., 1989). This is understandable because in the particle agglutination method, HIV antigens being on the beads “sandwich” HIV antibody.

As we mentioned above, it is acceptable to use 2 or more RDTs for initial screening to identify HIV infected individuals in resource-limited settings (Johnson et al., 2015). We envision that the test based on the UPA method for HIV antibody detection may be used for this purpose. Other UPA-based tests such as those for the detection of HIV-1/2 antibody and antigen (HIV-1/2 Ag/Ab Combo 4th generation test) can be used as screening tests and those for the differentiation of HIV-1 and HIV-2 antibodies can be used as confirmatory (supplemental) tests.

It is important to emphasize that the UPA method can be considered a platform technology and its future use is not specific to HIV diagnosis. Indeed, the UPA method can potentially be adapted to the study of the formation of HIV virion–antibody aggregates in the process of HIV virus neutralization. Vaccines based on such antibody-mediated viral aggregation are currently under development, as reported recently (Stieh et al., 2014) (Alexander et al., 2015). In addition, the UPA technology could potentially be utilized to increase the sensitivity of particle agglutination assays for detection of a wide range of biomarkers.

5. Conclusion

The UPA HIV antibody test presented here is performed as a single-step assay. The UPA method we have developed offers significant advantages over conventional HIV tests, including a short time of analysis, increased sensitivity, quantifiable results, and lower cost. We realize, however, that a clinical study including examination of hundreds clinical negative and positive samples is necessary for validation of the UPA method. This method is a step towards expanding the use of particle agglutination technology for detection of various markers and could be applied for increasing the sensitivity of other particle agglutination tests.

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Highlights

- An ultrasound particle agglutination (UPA) method is described.
- The UPA method uses dual mode ultrasound to reduce non-specific agglutination.
- The UPA method eliminates manual manipulation of samples.
- Optical detection allows accurate quantitative assessment of agglutination process.
- The UPA method is 100–500 times more sensitive than conventional rapid HIV tests.

Fig. 1. Configuration of the UPA device

(A) General view, and (B) architecture of the components, of the UPA device. The device is connected to a laptop computer via USB port. Insert in panel A shows the resonator cell (ultrasonic transducer) in which the samples are subjected to ultrasonic exposure and agglutination is monitored.

Fig. 2. The effect of single and dual ultrasound modes on the level of non-specific agglutination Images of negative and positive samples after 5 minutes of ultrasonic exposure: (A) using single mode ultrasound, and (B) using dual mode ultrasound. The positive sample is a positive control serum from Capillus rapid test kit diluted 1/400 in negative serum (Sigma).

Fig. 3. Comparison of analytical sensitivities of Capillus HIV rapid test and UPA method

(A) Pictures of Capillus HIV rapid test capillary slides: slides 1, 2 and 3 contain the positive control serum in dilutions 1/2, 1/4 and 1/8, respectively; slide 4 contains negative serum used for dilution (Sigma serum). Limit of Detection (LOD) is 1/4 diluted positive control serum. (B) UPA calibration curve prepared using serial dilutions of Capillus positive control serum in Sigma serum (Neg). Each data point (◆) shows an average and standard deviation value of triplicate measurements. LOD's for the conventional agglutination Capillus assay and UPA test are shown by arrows.

Fig. 4.
Analysis of clinical presumably negative samples by the UPA method.

Table 1

Parameters used for performing the UPA method.

Mode	Single-frequency mode			Swept-frequency mode		
Parameter	Frequency output, a. u.	Frequency, MHz	Duration, min	Frequency output, a. u.	Frequency range, MHz	Duration, min
Parameter value	750	13.1	1.5	550	12–22	3.5

Table 2

Statistical Parameters and Cut-Off Values Determined from Fitted PDF

Mean	Median	Mode	Standard Deviation	Cut-Off (method 1)		
178.46	118.85	52.71	199.90	($\mu + 2\sigma$)	($\mu + 3\sigma$)	($\mu + 4\sigma$)
				578.26	778.16	978.08

Table 3Comparison of limits of detection (LOD) of rapid tests and UPA method^a

Test Kit	Capillus™	Multispot™	Uni-Gold™
Conventional rapid test LOD	1/4	1/2	1/2
UPA method LOD	1/2040	1/230	1/160
Increase in analytical sensitivity	510-fold	115-fold	80-fold

^aThe UPA method was implemented using HIV antigen coated latex beads from the Capillus kit and dilutions of positive control serum from respective rapid test kits. The LOD's are expressed as dilutions of the positive control samples from corresponding test kits. Increases in analytical sensitivity of the UPA method over that of conventional rapid test are expressed as the ratio of LOD's of UPA to each of the rapid tests.