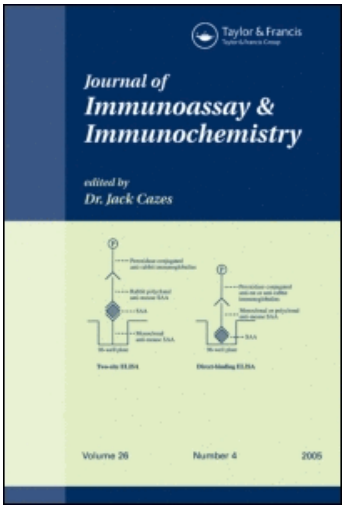

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ROTAVIRUS DETECTION USING ULTRASOUND ENHANCED LATEX AGGLUTINATION AND TURBIDIMETRY

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ABSTRACT

Application of a non-cavitating ultrasonic standing wave to suspended micro-particles brings the particles into close approximation and has been used previously to enhance the performance of several diagnostic agglutination tests. The sensitivity of rotavirus detection by ultrasound enhanced latex agglutination was compared with conventional test-card agglutination. Application of ultrasound gave a 32-fold improvement in the sensitivity of detection of rotavirus antigen in buffer compared with the test card method. A novel turbidimetric approach was used to measure agglutination occurring following the test-card procedure (in place of visual examination) and following exposure of commercial rotavirus latex reagents to a 4.5 MHz ultrasonic field (in place of microscopy). The sensitivity enhancement over the conventional method achievable through ultrasonic exposure was comparable whether agglutination measurements were made visually or turbidimetrically and demonstrates the potential for turbidimetry in combination with the ultrasonic method. Turbidimetry offers an alternative to visual assessment that may be more easily incorporated into automated systems.

(KEYWORDS: Ultrasound-enhanced latex agglutination, Rotavirus, Antigen detection, Turbidimetry)

INTRODUCTION

Non-cavitating ultrasonic standing waves are employed to manipulate suspended micro-particles in many biotechnological applications (1). Antibody coated diagnostic agglutination particles can be concentrated at preferred regions in an ultrasonic standing wave field to increase the sensitivity of analyte detection compared with standard agglutination techniques. Up to 1024-fold improvements in the sensitivity of antigen detection (2-3) and 32-fold test-time reductions (4) have been reported for a number of particle-based tests using ultrasound (1).

Rotavirus infection is a major cause of acute childhood gastroenteritis in the UK (5) and is a significant healthcare burden worldwide (6-7). For detection of rotavirus the gold standard of electron microscopy (EM) is costly, time consuming and labour intensive and enzyme linked immunoassay is a relatively lengthy procedure compared to latex agglutination. Since latex agglutination tests (LATs) are regarded as being less sensitive than EM, it is possible that if enhancements reported for other tests can be reproduced for rotavirus latex agglutination, then parity of performance with EM might be achievable. This preliminary study aimed to assess the feasibility of viral coat antigen detection by ultrasonic enhancement using a commercially available rotavirus LAT and compares the use of turbidimetry and light microscopy for measurement of agglutination.

MATERIALS AND METHODS

A commercially available rotavirus LAT (Slidex Rotakit, bioMerieux, UK) was used throughout. The test latex reagent (latex microparticles coated with

antibody specific for rotavirus antigen) was appropriately diluted in sample buffer provided with the kit. For turbidimetric examination, the latex reagent was diluted 1/2 in sample buffer for both conventional and ultrasonic methods. Kit control positive antigen was serially diluted in sample buffer to give an antigen dilution range from neat to 1/1024. Kit sample buffer was used as a negative control.

In the conventional method, 20 µl of test latex (either undiluted or at a 1/2 dilution) and 20 µl of sample were mixed on a kit reaction card to form a 10 mm diameter pool of reaction mixture. The card was rotated using an orbital shaker at 160 r.p.m. for two minutes. The reaction mixture was examined for agglutination using either (i) the naked eye in accordance with the manufacturer's instructions or (ii) turbidimetry to allow objective measurement of latex agglutination. For unaided visual observation of agglutination, the detection limits were determined in duplicate. For turbidimetry, a volume of 60 µl in total of reaction mixture was transferred (following rotation) using a 2 mm internal diameter glass capillary (Fisher Scientific, UK) to a plastic tube for measurement as described below.

In the ultrasonic method, 20 µl of test latex (diluted from 1/2 to 1/16) and 20 µl of test sample were mixed and immediately drawn into a 2 mm internal diameter glass capillary tube using an attached syringe. The droplet of reaction mixture within the capillary was exposed to ultrasound for 3 minutes on the axis of a tubular ultrasonic transducer (Morgan Matroc, UK) enclosing a reservoir of distilled water filled to the upper edge of the transducer (Figure 1) as described previously (2). RF voltage was applied using a commercially available wave-generator (Hameg Instruments, UK). An ultrasonic standing wave field of 4.5 MHz

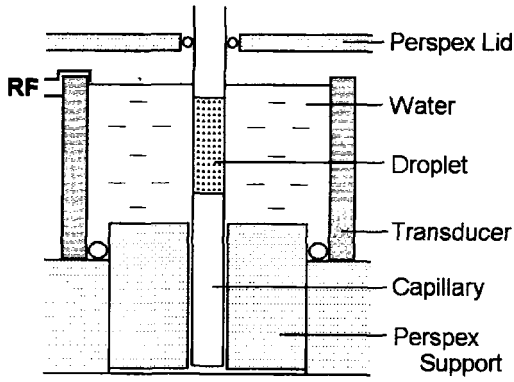

FIGURE 1. A section through the ultrasonic chamber showing, the tubular transducer (of dimensions 20 mm height, 31.5 mm internal diameter with a wall thickness of 1.9 mm), water for transmission of ultrasound, and the test sample within the on axis capillary. The capillary was centrally located by the O-rings shown at the lid and the perspex support.

was employed with a minimum voltage output of 15 volts peak to peak across the transducer. For visual observation of agglutination, the capillary was removed from the apparatus (following sonication) and the reaction mixture was expelled onto a solid non-absorbing surface (such as the test-card). The droplet was stirred 3-4 times with a mixing stick (supplied with the kit) to break up non-specific aggregates formed as a result of ultrasonic concentration (3). The droplet was loaded into a 200 μm pathlength cross-section microslide (Camlab, UK) by capillarity and examined for agglutination by microscopy (× 10 objective). The detection limits were determined in duplicate. For turbidimetry, the capillary was removed from the apparatus following sonication and the reaction mixture was expelled into a plastic centrifuge tube to give a final volume of 60 μl for subsequent measurement as described below.

An adapted turbidimetric method was used to measure agglutination. The test sample (transferred to a centrifuge tube following treatment) was centrifuged at 5 g for 5 min. to remove agglutinates formed in each procedure. Thirty microlitres of the supernatant was added to 70 µl of kit sample diluent in a flat bottomed microwell plate and absorbance was read at a wavelength of 405 nm using a 'Multiskan Plus' plate reader (Titertek, Eflab, Finland). A wavelength of 405 nm was closest to the size of the particle and therefore constitutes the most discriminatory wavelength. It was necessary to use wide-bore channels for aspiration and elution of samples as the shearing forces generated using conventional micropipette tips can dissociate specifically bound particles. Conventional and ultrasonic tests were performed in quadruplicate for turbidimetric measurements.

RESULTS

In the test-card format, with visual inspection, the lowest antigen dilution at which agglutination was still discernible was 1/16 using undiluted test latex. Particle dilution reduced the sensitivity of antigen detection by conventional agglutination. However, in the ultrasonic method, the lowest antigen level at which agglutination was observed microscopically was at a 1/512 dilution of antigen using a 1/8 dilution of test-latex.

To compare visual interpretation with turbidimetry it was necessary to use a 1/2 dilution of test latex reagent for both test card and ultrasonic methods (in the turbidimetric method used here, measurements were not possible at latex dilutions

of 1/4 or less). The absorbance readings for the negative control samples determined for the ultrasonic and conventional methods differed due to ultrasonically induced aggregation of microparticles. The difference in absorbance was therefore used as a correction factor which was summed with each absorbance measurement for sonicated samples. Corrected absorbances determined ultrasonically for all serial dilutions of positive control antigen and negative controls were plotted against results found using the conventional LAT in Figure 2 (error bars represent +/- one standard deviation from the mean in positive samples and two standard deviations below the mean in negative samples). Negative cut-off values for both methods were defined as 2 standard deviations below the mean of each respective negative control. The mean absorbances (corrected absorbance for sonicated samples) were paired between conventional and ultrasonically treated methods and subjected to one-tailed paired t-testing yielding a *P*-value of 0.002. The statistically determined cut-off value i.e. the detection limit, for ultrasonically enhanced specimens was at a 1/32 dilution of antigen compared with a 1/2 antigen dilution for the conventional LAT, representing a 16-fold improvement in test sensitivity. The antigen detection limits determined by visual examination of agglutination following conventional and ultrasonic tests (using a 1/2 reagent dilution) are compared with those achievable for both methods through the use of turbidimetry in Table 1.

DISCUSSION

Ultrasonic concentration of diagnostic micro-particles (typically 0.2-1 μm diameter) causes close approximation of particles at submillimetre distances

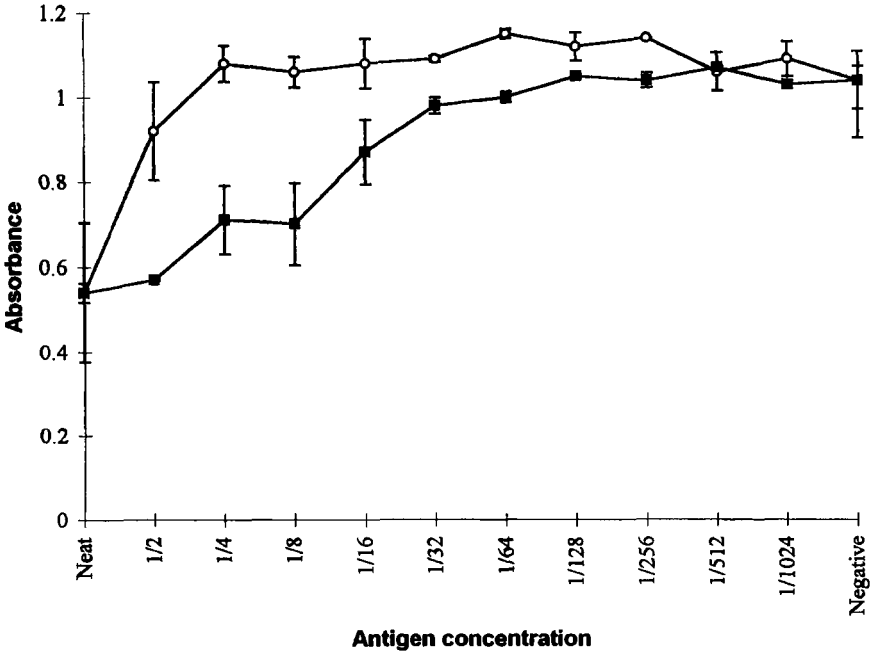

FIGURE 2. Absorbances determined for serial dilutions of positive control antigen with the conventional LAT (O) and ultrasonic test (■). Corrected absorbances (0.155 correction factor) are presented for each ultrasonic test.

TABLE 1

Comparison of Antigen Detection by Conventional LAT and the Ultrasonic Method using either Visual Assessment or Turbidimetric Analysis.

	Lowest Dilution of Antigen at which Agglutination was Detectable	
Method	Visual Assessment	Turbidimetry
Conventional LAT	1/4	1/2
Ultrasound-Enhanced Latex Agglutination	1/32	1/32
Sensitivity Enhancement over Conventional LAT	× 8	× 16

throughout the suspension (at positions of minimum acoustic potential energy), increasing particle contact and hence aggregation of particles (1). Application of shearing forces following sonication (simple manual stirring prior to microscopy) dissociates any clumps formed unless particles are bridged by antibody-antigen interactions. In the turbidimetric method, sonicated reaction mixtures were centrifuged and transferred to a microplate reader and no shearing force other than that generated during sample handling was applied. The measure of agglutination is reflected in the number of non-agglutinated particles remaining in the supernatant where centrifugation removes the larger agglutinates formed by particle cross-linking. Correction for ultrasound induced non-specific aggregation before statistical analysis supports the argument for greater sensitivity due to increased antigen-antibody cross-linking.

In this preliminary exercise in rotavirus diagnosis, the sensitivity limit achievable by visual observation using test-card agglutination or ultrasound-enhancement with optimal dilutions of latex (undiluted and diluted 1/8 respectively) was lower than with the turbidimetric procedure employed. Ultrasound treatment using optimal dilutions of test latex reagents gave a 32-fold improvement in test sensitivity over the conventional test-card method when agglutination was assessed by visual means. Table 1 shows that comparable sensitivity enhancements were achievable using ultrasound over conventional tests whether visual or photometric means were used to measure agglutination. Equal latex particle dilutions were used for turbidimetry and for visual assessment of agglutination so as to facilitate accurate photometric comparisons of agglutination occurring following test-card

rotation or sonication. The sensitivity of ultrasonic tests with photometric agglutination measurement could be improved with optimal dilution of latex particles. Non-intentional shearing forces generated during sample handing may have contributed to reduced sensitivity but resulting physical stresses could ultimately be harnessed so as to remove non-specific aggregates formed following ultrasonic exposure.

This is the first study coupling the ultrasonic immunoassay with a turbidimetric technique and demonstrates the potential of photometry for measurement of agglutination. Previous studies of ultrasound enhancement have relied on naked-eye assessment (4), microscopic assessment (2) or image analysis of observer selected microscope fields (8), all methods being prone to subjective bias (8) and not easily amenable to automation. Subjectivity associated with visual inspection of agglutination can be overcome through operator experience and is only an issue at threshold antigen levels where the degree of residual background aggregation may influence the operators interpretation of microscopic fields (9).

Absorbance determinations allow quantitative estimation of antigen level while compensating for ultrasound induced aggregation. This study reproduces, for rotavirus latex agglutination, previously reported enhancements observed with other ultrasonic tests for detection of bacterial antigen (10-11). Given that the detection of rotavirus by standard LATs has been reported to be as low as 66% (12-14) when compared with EM, a translation of this 32-fold improvement from purified antigen preparations to clinical samples could bring the test sensitivity of latex agglutination to parity with EM. In addition, the ultrasonic immunoassay can provide results in

less than 30 minutes from receipt of an unprocessed faecal specimen, offering considerable time advantage in comparison to EM. It is conceivable that improved diagnostic methods could assist in epidemiological studies and in the evaluation of introduced vaccine strategies (6-7), however, the sensitivity and specificity of antigen detection in clinical samples remains to be addressed.

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