



Ultrasound wave-mediated enzyme-linked immunosorbent assay technique

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ABSTRACT

With the increasing burden of infectious diseases, it has become important to develop a rapid ELISA that could facilitate early diagnosis. Herein, we have shown that ultrasound waves can dramatically reduce the ELISA timing without losing its specificity or sensitivity. Ultrasound-mediated ELISA was best achieved on an activated microtiter plate which was able to covalently bind antigen or antibody in 10 min when subjected to ultrasound waves in a sonicator bath having a temperature of 37 °C, operating at a frequency of 40 KHz and an output power of 120 W. Blocking, antibody binding and secondary antibody–enzyme conjugate binding were also accomplished in 10 min each in the sonicator bath under similar conditions. The validation of SELISA method was demonstrated by detecting IgE in allergic patient's sera. Total IgE detection by 40 min-SELISA method gives similar absorbance value to that obtained by 20 h conventional or 3 h-HELISA procedure. As SELISA method can detect IgE even at the serum dilution of 1/50 (v/v) on photoactivated surface it could be significantly useful to confirm false negative cases. The inhibition assay ruled out the possibility of any cross reactivity or non-specific binding. As SELISA procedure is sensitive, specific and reproducible (intra- and inter-assay CVs were 9.65% and 8.47%) it could be an excellent alternative to conventional ELISA or HELISA procedures.

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1. Introduction

A number of serological tests have been used for the detection of antibodies in patients suffering from allergy, fungal [1], bacterial [2], parasitic [3] and invasive diseases like HIV and hepatitis. Among them enzyme-linked immunosorbent assay (ELISA) technique appears to be the most popular due to its sensitivity, specificity, accuracy and reproducibility. However, major drawback of ELISA technique is its time consuming procedure. Since the invention of this technique in 1971 by Engvall and Perlmann [4], lots of efforts have been made to improve ELISA technique by incorporating several approaches including use of monoclonal antibodies [5–7], sensitive tags such as fluorescent tags [8], chemiluminescent [9], radioactive moieties [10] or cofactors [11]. Enhanced ELISA values were also shown by binding antigen or antibody covalently [12–15] onto the microtiter plate. Improved polystyrene microtiter plates have considerably enhanced specificity, sensitivity, rapidity, and reliability of the test. Advancement of ELISA technique using improved microtiter plates needs special attention as this approach does not require costly chemicals and reagents. The surface modifications of the wells of polystyrene microtiter plate were carried out by different methods such as grafting of glycidylmethacrylate [16], introducing carboxylic group [17,18] or amino group [19] or incorpo-

rating PVDF membrane [20]. These activated surfaces were reported to yield more efficient ELISA than the untreated surfaces. Recently, Nahar et al. have activated polystyrene plate using a photolinker, 1-fluoro-2-nitro-4-azidobenzene (FNAB) by light [21]. Such photoactivated microtiter plates shortened the ELISA timing to 7.5 h with results comparable to that of conventional ELISA carried out in 18 h [22]. Bora et al. further reduced ELISA timing to 3 h by heat-mediated ELISA (HELISA) procedure on such photoactivated surface [23]. Later Kumar et al. [24] detected cell adhesion molecules (CAMs) on human umbilical vein endothelial cells (HUVECs) at elevated temperature. Recently, Sharma et al. [25] detected autoantibody in rheumatoid arthritis patients by HELISA. In HELISA each step of antigen binding, blocking, antibody binding and antibody–enzyme conjugate were carried out at 50 °C in 45 min, 40 °C in 40 min, 50 °C in 45 min and 50 °C in 40 min, respectively.

In this communication, we have for the first time demonstrated the use of ultrasound waves, an alternate source of energy, in reducing ELISA timing for antibody detection. The procedure has dramatically reduced the total time required for ELISA to 40 min with the retention of sensitivity and specificity.

2. Experimental

2.1. Chemicals

Anti-human IgG, anti-human IgE, bovine serum albumin (BSA), human IgG, anti-human IgG–HRP conjugate, anti-human IgE–HRP

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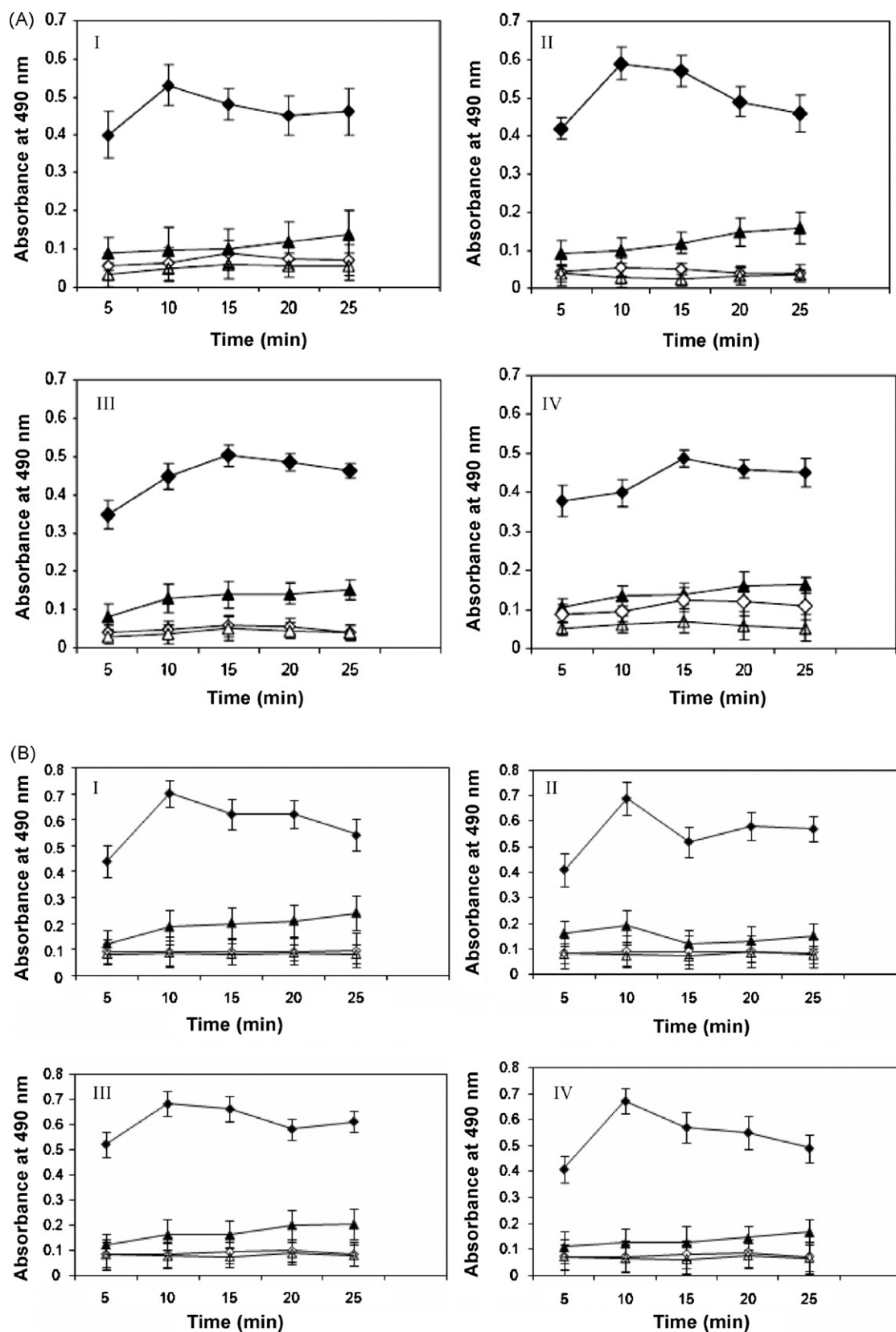


Fig. 1. Optimizations of sonication time for anti-human IgG immobilization, (I), blocking (II), human IgG binding (III) and anti-human IgG peroxidase binding (IV). In each of the optimization experiments the subsequent steps were carried out by conventional ELISA and assayed colorimetrically. Dark and hollow symbols represents positive and negative control sera while (◆) and (▲) represent activated and untreated wells. For each step of SELISA ultrasound power was optimized by doing the steps at 110 W (A), 120 W (B) and 130 W (C).

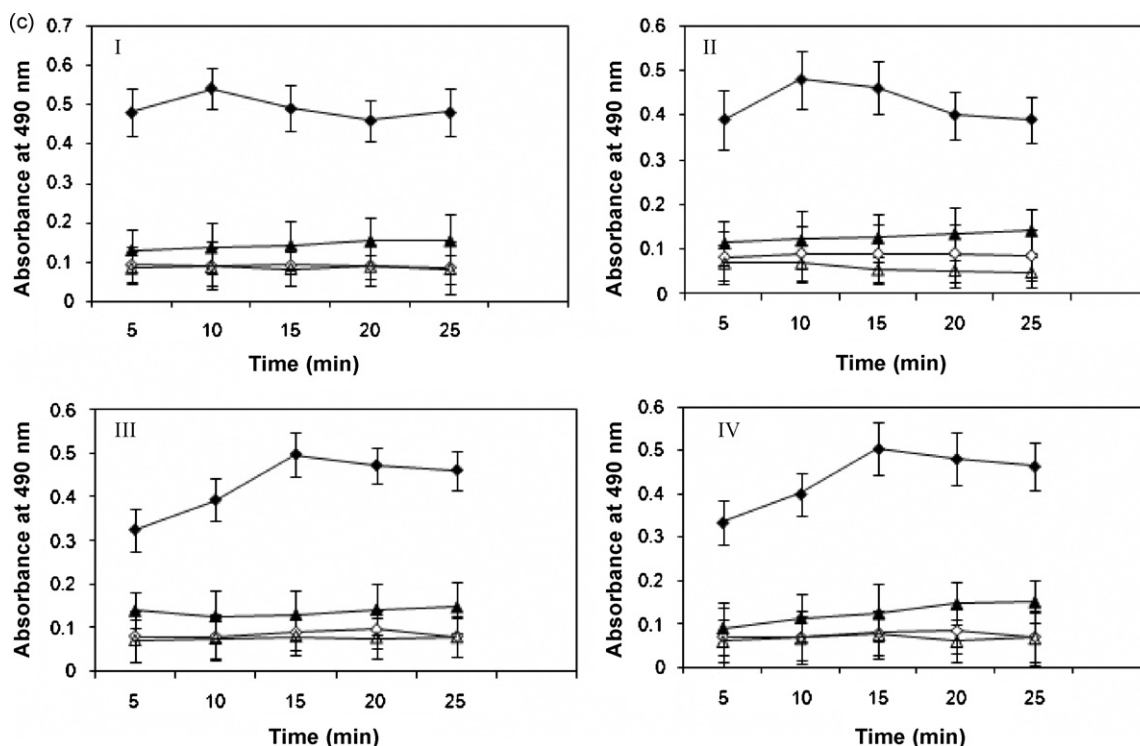


Fig. 1. (Continued).

conjugate and o-phenylenediamine dihydrochloride (OPD) were purchased from Sigma (USA). IgE kit was procured from Bethyl Laboratories, USA. 1-fluoro-2-nitro-4-azidobenzene (FNAB) was prepared from 4-fluoro-3-nitro aniline (Aldrich, USA) by a simple diazotization reaction [26]. Polystyrene microtiter plates (Greiner labortechnik, Germany) were activated by FNAB as described by Nahar et al. [21]. Ultrasound waves were generated in a sonicator bath (Elma, Germany) operating at a frequency of 40 KHz and output power ranging from 110 to 130 W. Temperature of the sonicator bath was maintained at $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$ by putting ice or cold water in the sonicator bath. All the experiments were carried out with activated and untreated plates. Washing buffer was prepared by adding 0.1% Tween-20 and 0.85% NaCl to 0.01 M phosphate buffer (PB), pH 7.2. Concentration of anti-human IgG, human IgG and anti-human IgG–HRP conjugate were optimized by checker board titration. For IgE detection sera from allergic patients was used.

2.2. Detection of anti-human IgG by conventional ELISA

Anti-human IgG ($0.125\text{ }\mu\text{g}/100\text{ }\mu\text{L}$ of 0.01 M PB/well) was immobilized in the wells of a microtiter plate by incubating at 4°C for 13 h (overnight). After blocking with 2% BSA solution at 37°C for 1 h, wells were incubated with human IgG ($0.125\text{ }\mu\text{g}/100\text{ }\mu\text{L}$ of 0.01 M PB/well) for 3 h at 37°C . Then peroxidase conjugated goat anti-human IgG ($100\text{ }\mu\text{L}$ of 1:5000 dilution) was added and incubated for 3 h at 37°C . After each step the wells were washed with washing buffer. Color development was carried out by adding $100\text{ }\mu\text{L}$ of substrate-dye buffer (12 mL of citrate buffer, pH 5; $5\text{ }\mu\text{L}$ of 30% H_2O_2 and 5 mg of OPD). After 5 min, $20\text{ }\mu\text{L}$ of stop solution (5% H_2SO_4) was added to each well and absorbance was recorded at 490 nm.

2.3. Ultrasound-mediated immobilization of goat anti-human IgG

Anti-human IgG solution ($0.125\text{ }\mu\text{g}/100\text{ }\mu\text{L}$ of 0.01 M PB/well) was poured into the activated and untreated wells of microtiter plates and floated on the sonicator bath. Ultrasound waves were

generated at a frequency of 40 KHz and an output power of 110 W. The plates were sonicated for 5, 10, 15, 20, 25 and 30 min, respectively. After the stipulated time the plates were washed and the subsequent ELISA steps were carried out by conventional method as mentioned in Section 2.2.

2.4. Ultrasound-mediated blocking of goat anti-human IgG immobilized plate

Anti-human IgG was immobilized by sonication in 10 min as mentioned in Section 2.3. Sonication-mediated blocking was optimized by adding 2% BSA ($200\text{ }\mu\text{L}/\text{well}$) and subjecting the plates to ultrasound waves for 5, 10, 15, 20, 25 and 30 min, respectively at 110 W. Absorbance was recorded after performing the remaining ELISA steps conventionally as described in Section 2.2.

2.5. Time dependent binding of human IgG to goat anti-human IgG immobilized plate

Immobilization of goat anti-human IgG in the wells of microtiter plates and subsequent blocking were carried out by sonication in 10 min each. Human IgG ($0.125\text{ }\mu\text{g}/100\text{ }\mu\text{L}$ of 0.01 M PB/well) was loaded to these blocked wells and sonicated at 110 W for 5, 10, 15, 20, 25 and 30 min, respectively. After washing, remaining ELISA steps was carried out by conventional method.

2.6. Time dependent binding of goat anti-human IgG peroxidase to human IgG bound to the microtiter plate

Goat anti-human IgG binding, blocking and human IgG binding were carried out in the wells by ultrasound waves in 10, 10 and 15 min, respectively at 110 W. Goat anti-human IgG peroxidase ($100\text{ }\mu\text{L}$ of 1:5000) were added to the wells and subjected to ultrasound waves at 110 W for 5, 10, 15, 20, 25 and 30 min, respectively. Color development was carried out as described.

2.7. Optimization of time for SELISA steps at an output power of 120 W

The experiments (Sections 2.3–2.6) were carried out similarly except that an output power of 120 W was used instead of 110 W. For each ELISA step (except goat anti-human IgG immobilization step) optimized sonication timing of preceding step was used.

2.8. Optimization of time for SELISA steps at an output power of 130 W

The experiments (Sections 2.3–2.6) were carried out similarly except that an output power of 130 W was used instead of 110 W. For each ELISA step optimized sonication timing of preceding step was used.

2.9. Detection of human IgG by conventional ELISA, HELISA and SELISA

Human IgG was detected by conventional ELISA (4 °C, 13 h/37 °C, 1 h/37 °C, 3 h/37 °C, 3 h), HELISA (50 °C, 45 min/40 °C, 40 min/50 °C, 45 min/50 °C 40 min), SELISA (10 min for each step at 37 °C) and control ELISA carried out at the same temperature and time (37 °C in 40 min) as in SELISA but without sonication.

2.10. Standard curves by conventional and SELISA procedure

In the SELISA procedure, 100 μ L/well goat anti-human IgE (0.125 μ g/100 μ L of carbonate buffer) was immobilized in activated wells followed by blocking with BSA. The wells were then loaded with 100 μ L of IgE standards as per the supplier's instructions in a double-dilution series (1000, 500, 250, 125, 62.5, 31.25 and 15.34 ng mL⁻¹) and sonicated. Goat anti-human IgE peroxidase (1/15,000) solution was added to these wells. Each step was performed by sonication at an output power of 120 W in 10 min. After washing the plates were assayed for peroxidase activity. A conventional ELISA for IgE detection was carried out in 20 h (4 °C, 13 h; 37 °C, 1 h; 37 °C, 3 h; 37 °C, 3 h).

2.11. Sensitivity study (limit of detection) of SELISA technique

Anti-human IgE (0.125 μ g/100 μ L of carbonate buffer) was immobilized into activated polystyrene wells, blocked and pooled patients sera in different dilutions (1/2, 1/10, 1/50, 1/250 and 1/500 v/v) were added to the wells to bind with anti-human IgE. Lastly, conjugate binding was carried out and absorbance was recorded after adding substrate-dye buffer. Ultrasound waves at an output power of 120 W were used for 10 min for each of these steps. Simultaneously, these steps were carried out by HELISA as described in Section 2.9.

2.12. Inhibition study

Ultrasound-mediated solid phase was prepared by immobilizing goat anti-human IgE (0.125 μ g/100 μ L of 0.01 M carbonate buffer) in the wells of an activated plate followed by blocking. Separately, a double-dilution series of goat anti-human IgE (0–2 μ g mL⁻¹ of PB) was taken in microcentrifuge tubes to which 100 μ L of pooled patients sera (1/50 v/v dilution) was added and incubated for 1 h at 37 °C. From these 100 μ L/well of the mixture was added to the solid phase and sonicated for 10 min. Goat anti-human IgE peroxidase (1/15,000) solution was added to these wells and sonicated for 10 min. Sonication steps were carried out at an output power of 120 W. After washing the plates were assayed for peroxidase activity.

2.13. Validation of SELISA technique

Total IgE in patients' sera was determined by SELISA technique in 40 min at an output power of 120 W. The sonication-mediated enzyme-linked immunosorbent assay was validated for sensitivity, precision and specificity. The sensitivity is expressed as a limit of detection (LOD). Reproducibility and precision of the assay were calculated. The variations between replicates from well to well (intra-assay) and plate to plate (inter-assay) were determined. Specificity was evaluated by inhibition experiments. Correlations between SELISA and conventional ELISA tests were determined by Deeming procedure. All statistical analyses were done with web based statistical software.

3. Results and discussion

Conventionally, ELISA procedure was carried out through adsorption on a polystyrene microtiter plate customarily known as ELISA plate. However, SELISA technique is best achieved on a polystyrene plate that binds an antigen or antibody by covalent bonding. To enable covalent binding, a polystyrene microtiter plate was activated by a photolinker, 1-fluoro-2-nitro-4-azidobenzene (FNAB). Activation was done by coating the wells of the microtiter plate with FNAB and exposing them to UV light of 365 nm wavelength as reported earlier [21,22]. In SELISA technique, all the ELISA steps were carried out by ultrasound waves generated in a sonicator bath operating at a frequency of 40 KHz and an output power ranging from 110 to 130 W. Sonication power for SELISA steps were optimized by carrying out each step at a fixed time at variable output power. Similarly, sonication time for SELISA steps were optimized by carrying out each step at a fixed output power at variable time. After optimization subsequent steps were carried out by conventional ELISA procedure. Covalent immobilization of goat anti-human IgG onto the activated polystyrene plate, blocking, human IgG binding and anti-human IgG peroxidase binding were best achieved by subjecting the plate to ultrasound waves for 10, 10, 15 and 15 min, respectively at 110 W (Fig. 1A). Same optimized time for these steps were obtained when they were carried out at 130 W (Fig. 1C). However, when these steps were carried out at 120 W of ultrasound power, the corresponding optimized time were 10 min for each step (Fig. 1B). Thus, at an optimum output power of 120 W, optimum time for covalent immobilization of goat anti-human IgG, blocking, human IgG binding and anti-human IgG peroxidase binding were 10 min each. Thus, total time for SELISA was 40 min only. Nevertheless, 5 min sonication in each step gave an absorbance value that was sufficient to detect an antibody. Untreated surface however, did not show any appreciable immobilization (Fig. 1A).

We have further compared SELISA with conventional ELISA and HELISA. Absorbance value obtained in SELISA was akin to that of conventional ELISA and HELISA. To find out whether ultrasound waves indeed accelerated ELISA, we also performed ELISA at 37 °C but without applying sonication. Control experiment carried out without sonication showed very low absorbance values on both treated and untreated surface, thus confirming the role of ultrasound waves as an accelerator of ELISA (Fig. 2).

Further we extended the SELISA method by detecting IgE in human sera on the photoactivated microtiter plate. Due to the very low amount of IgE in blood sera the conventional ELISA procedure requires extended times of incubation. However IgE too can be well detected in very short time by sonication. This fact was verified by standard curve prepared by doing ELISA by conventional and SELISA method (Fig. 3).

Sensitivity of the present method was determined to find out the maximum dilution of the sera at which IgE could be detected. Detection of IgE at serial dilutions of the pooled serum sample of

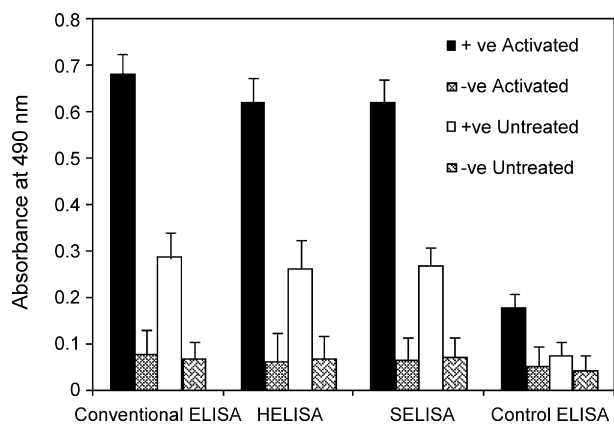


Fig. 2. Comparison between 20 h-conventional ELISA, 2 h 50 min-HELISA, 40 min-SELISA and 40 min-control ELISA. Control ELISA was carried out in the same temperature and time as in SELISA but without sonication. The error bars represent the standard errors of the means.

allergic patients on the activated microtiter plates was performed by SELISA method. Parallely, IgE detection was also carried out by HELISA method. Results of SELISA method were akin to that of HELISA method indicating that SELISA could be a good substitute for HELISA for detection of IgE even at higher dilution. The limit of detection (LOD) of IgE was at the serum dilution of 1:50 (Fig. 4). This is significantly important to confirm false negative cases.

We have further evaluated the specificity of the assay by inhibition experiment. Anti-human IgE preincubated with patient sera showed an inhibition of binding of antibody in a dose dependent manner. This inhibition assay confirmed that non-specific binding or cross reactivity did not occur in SELISA. Moreover, with decreasing amounts of free antigen, the inhibition was reduced on both the untreated and activated surfaces in a similar pattern which shows that the covalent immobilization on activated surface does not affect the antigenic site during immobilization (Fig. 5).

Intra-assay and inter-assay precisions were also studied. Intra-assay precision was evaluated by the variation of the measurement of IgE from well to well ($n=36$) (12 wells/sample) and inter-assay precision was obtained from the variation from different plates ($n=10$) on different days. The coefficient of variation (CV), was calculated as $CV = SD/mean \times 100\%$. The intra-assay CV was 9.65% and inter-assay CV was 8.47%.

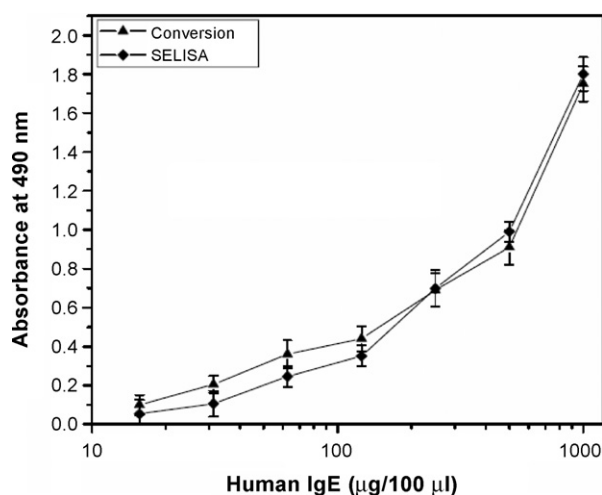


Fig. 3. Determination of total IgE by standard curves obtained by carrying out SELISA (◆) and conventional ELISA (▲) techniques.

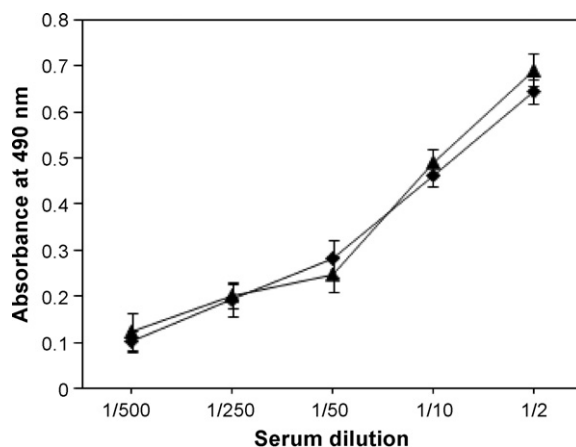


Fig. 4. Sensitivity study of SELISA technique. Human IgE is detected in patient sera at different dilutions by SELISA (◆) and HELISA (▲) techniques on activated polystyrene microtiter plate.

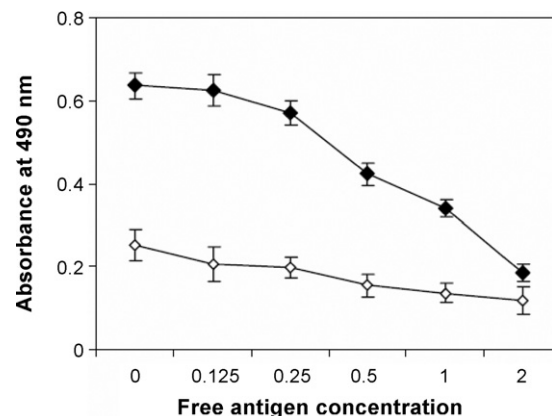


Fig. 5. Inhibition of IgE binding to the antigen immobilized on an activated (◆) and untreated (◇) plates using different concentrations of free antigen ($2-0 \mu\text{g mL}^{-1}$). A 1:50 (v/v) diluted serum and anti-human IgE peroxidase conjugate (1:15,000 v/v) was used in the assay.

The antibody measured by SELISA method shows good correlation with those determined by the conventional ELISA. The slope and intercept for this correlation analysis were 1.026 and 0.0096 and $R=0.9564$. Deeming regression analysis (including 95% confi-

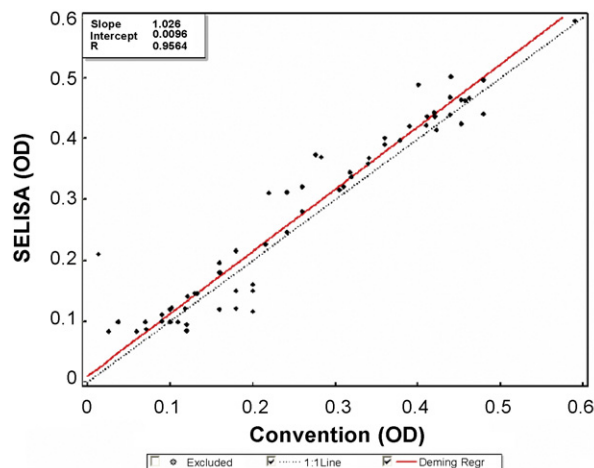


Fig. 6. Correlation between SELISA and conventional ELISA for IgE detection by Deeming regression analysis (solid line). Dashed line represents an ideal comparison ($x=y$).

dence interval for slope setting intercept as 0.0062) reveals that there is no significant difference between O.D. values analyzed by two different ELISA procedures. The equation is $y = 1.026x + 0.0096$. The correlation is significant at the level of 0.01 (Fig. 6).

4. Conclusion

Our study highlights the potential of ultrasound waves in carrying out the important immunoassays like ELISA. SELISA carried out by sonication on an activated plate requires only 40 min without involving any costly monoclonal antibody or sensitive tag. Since, SELISA technique is able to detect antigen even at higher dilutions, the technique would be beneficial to confirm false negative cases. Besides, the method is precise and reliable, thus it can be a good substitute for conventional immunoassay procedures widely used in clinical and research laboratories.

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