Rapid Detection System for Hepatitis B Surface Antigen (HBsAg) Based on Immunomagnetic Separation, Multi-Angle Dynamic Light Scattering and Support Vector Machine

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ABSTRACT Hepatitis B virus (HBV) is a significant public health problem worldwide. Hepatitis B surface antigen (HBsAg) is the principle marker for laboratory testing of HBV, but the rapid identification of HBsAg is challenging in a resource-limited setting. Antibodies to HBsAg (Anti-HBs) levels are measured as markers for an immune response to vaccination as well as for decision making for specific treatment against Hepatitis-B. This research developed a prototype for the rapid detection of HBsAg using immunomagnetic separation, dynamic light scattering, and support vector machine. Magnetic beads coated with polyclonal anti-HBsAg were used to isolate HBsAg from the sample. The performance characteristics of quantitative real-time detection of HBsAg were characterized under optimized conditions. Twelve photodetectors were arranged on four concentric curvatures at different angles. The photodetectors were positioned around the sample flask in forward direction. The prototype acquires the real-time laser scattering light from the sample, and the noise was removed. The power spectral features were extracted from the acquired signal. Support vector machines (SVM) were used for training a classification algorithm by using extracted features. The overall classification accuracy for the identification of HBsAg was 87.7%. The HBsAg detection test was also performed on 20 serum specimens, with 10 serum samples were positive for HBsAg and 10 were healthy control subjects. The test had a dynamic range of 98.86 IU/mL to 3163.5 IU/mL. Results of HBsAg detection agreed completely with those of conventional Chemiluminescence Immunoassay (CLIA). In conclusion, the proposed HBsAg detection method can differentiate the sample that contains HBsAg enriched IM beads and blank IM beads.

INDEX TERMS HBsAg detection, immunomagnetic separation, laser light scattering, support vector machine.

I. INTRODUCTION
Hepatitis B virus (HBV) is the most common cause of liver disease worldwide. HBV can be transmitted parenterally, perinatally, and sexually, as well as through household contact or unsafe injections. Chronic infections acquired during childhood lead to higher morbidity and mortality [1], [2]. The envelope protein of HBV is the Hepatitis B surface antigen (HBsAg). It is present in the serum of infected individuals, and the identification of HBsAg levels is essential...
to evaluate treatment response. HBsAg appears in serum at the early stages of HBV infection and can be detected 2 to 8 weeks before biochemical indications of liver dysfunction and jaundice. If HBV is not identified in the first six months of infection, then the infected person may be a chronic HBV carrier; spontaneous clearance is very unlikely. Therefore, early-stage screening of HBsAg level is vital but requires a good detection limit for low viral loads [3], [4].

Various methods have been developed for detecting serum HBsAg levels such as chemiluminescent immunoassay (CLIA), immuno polymerase chain reaction (iPCR) method, and enzyme-linked immunosorbent assays (ELISA) [5]–[7]. While these methods have excellent sensitivities but they are very expensive and require sophisticated instrumentation. These methods are also not suitable for emergencies. CLIA has limited test panels and high costs [8], while iPCR has been used widely for genomic studies, but it can be easily contaminated [9]. ELISA involves different time-consuming sample preparation steps and is expensive; these steps can affect the results if the parameters are not adjusted correctly [10], [11]. Further, the literature study shows that extensive research has been carried out by using a machine learning approach to classify HBsAg for the identification of HBV. Yuan et al. investigated five different classifiers for the HBV detection by using the acquired spectra from MALDI-TOF MS [12]. Runmin et al. constructed and compared machine learning algorithms with the FIB-4 score for the clinical prediction of HBV [13]. Xiaolu et al. applied different machine learning algorithms on the clinical data for predicting HBsAg seroclearance [14]. These methods and techniques are limited to the development of an algorithm only. Thus, there is a need for rapid and economic systems for the detection of HBsAg. The need for meeting these requirements has led to extensive research for improvising simple methods that reduce the overall cost and makes the process very simple.

This work uses immunomagnetic (IM) beads for the separation of HBsAg, and the classification of laser light scattering patterns based on support vector machine (SVM). The light scattering has been used widely in many biomedical devices to predict the physical and chemical characteristics of the sample [15]–[17]. The proposed system for pathogens identification is based on the basic principle of laser light scattering from the IM beads. When the laser beam passes by the sample, the surrounding photodetectors convert the acquired scattered light into a voltage waveform. The laser light scattering includes static laser scattering and dynamic laser scattering, whereas dynamic laser scattering (DLS) considered as a recognized configuration for particle sizing. The dynamic scattering can be estimated by basic setup; a laser is focused on the sample suspension. Most of the light passes through the sample, while some portion of the light is scattered. The scattered light detected at different angles classifies the sample based on the classification algorithm. Dynamic light scattering provides more information about the properties of materials based on the single scattering events.

Whereas, the multi-angle dynamic light scattering (MDLS) collects data from various scattering angles to analyze the data simultaneously. The principle of MDLS is based on the analysis of temporal fluctuations of scattered light from the particles in liquid. MDLS provides more information about the scattering light, robust and can increase the accuracy of system as compared with single-angle DLS [18], [19]. First, the HBsAg was separated out from the sample by immunomagnetic separation. IM beads are often used to concentrate and isolate target antigens from the sample [20], [21]. The IM separation method can concentrate target antigens from a large sample volume; unwanted agents were not collected. After immunomagnetic separation, the sample was used to acquire the data waveform of laser light scattering. After the removal of noise from the waveform, it was processed further to extract the power spectrum features for training SVM model for data classification. The trained SVM model was used as a classifier for the classification of testing samples by using power spectral features from the laser light scattering. The results were verified by using cross-validation. Fig. 1 illustrates the overall detection process which is divided into three parts; immunomagnetic separation, sample preparation and detection process. The approach of laser light scattering from the IM beads for target HBsAg is promising for rapid detection due to its high selectivity, easy manipulation, no pretreatment of sample, and no need for well-trained staff. However, applications of this approach for HBsAg detection have not been reported to the best of our knowledge.

The remainder of this paper is as follows: Section II describes the immunomagnetic separation of the sample...
and gives a detailed description of the clinical prototype. Thereafter, Section III provides the evaluation of optimum conditions for experiments. Section IV provides detailed information on the development of an algorithm based on power spectral features and support vector machine. Section V presents the experiments performed on clinical samples for system validation. Section VI provides detailed experimental results. Finally, conclusions are provided in Section VII.

II. MATERIALS AND METHODS

A. IMMUNOMAGNETIC SEPARATION OF HBsAg

HBsAg purified protein (10^4 ng/mL) were obtained from Shanghai Chaoyan Biotechnology Co. Ltd. (Shanghai, China). The anti-HBsAg-coated paramagnetic microparticles (IM beads) about 4 μm were obtained by using the ARCHITECT HBsAg Reagent Kit (Abbot, Ireland). The IM beads were used for isolating HBsAg from a fluid sample. The IM beads were diluted (1.6 × 10^4 ng/mL) by mixing 10 μL of the sample in 10 mL of distilled water and then washed with washing buffer (phosphate buffered saline, pH 7.4) before use.

Initially, 1 mL of a diluted sample containing HBsAg was mixed with 40 μL of IM beads and incubated at room temperature for 30 minutes. IM beads combine the HBsAg through the antigens on the beads. Then, these IM beads were concentrated by magnetic separator after incubation. The supernatant of the whole sample was poured out and HBsAg enriched IM beads were isolated. 1 mL of PBS was added to wash the isolated beads and separated from the solution by using magnetic separation. The washing and separation steps were performed three times. Separated IM beads were then mixed with 10 mL distilled water and were used to estimate laser light scattering.

B. MULTI-ANGLE DYNAMIC LIGHT SCATTERING SYSTEM

The basic principle of a given system is to measure the scattering of light from the sample. The prototype for measuring laser light scattering from the particles consists of three parts: a) laser light (λ = 660 nm), b) twelve photodetectors, and c) the data acquiring system. The photodetectors were arranged next to the sample holder to acquire forward light scattering. The clinical prototype including laser device was developed by the Chinese Academy of Sciences, Nanjing Institute of Advanced Laser Technology. The laser used in the system has a wavelength of 660 nm with a power rating of 150 mW, tested by using THORLABS power measuring instrument PM320E and S130C. The laser light of 660 nm was used because the wavelength of light should be less than the size of a particle to ensure that higher scattering occurs. A three-axis positioning system can adjust the position of laser light; in horizontal X and Y direction, and vertical Z direction. The positioning system helps in focusing the laser beam towards the center of the sample.

A small round bottom flask made of silica glass was used for performing experiments. The bottom flask with a length of 6.8 cm and a bottom diameter of 3 cm was manufactured by Hefei Celo Measure & Control Technology Co., LTD. The light absorbance property of glass was tested using SHIMADZU UV spectrophotometer (UV-1800), the light absorbance was nearly zero at the range of 500-700 nm wavelength. The prepared testing sample was placed in the round bottom flask for further experimentation. When the laser beam passes by sample flask, the scattering of light occurs in all directions depending on the size and shape of particle. Mie scattering theory describes the scattering of light by homogeneous particles when the particle size is comparable or larger than the wavelength of light being used [22]. The Mie plot depicts the scattering intensity of laser light based on the size and morphology of particles [23], [24]. Maximum light scattering occurs in forward direction with higher intensity. By using the photodetectors surrounding the sample in all direction, increases the complexity of system and need more computational power for classification algorithm. Therefore, Mie plot was used to optimize the position of photodetectors in forward direction. Using more channels give more information about the light scattering as compared with a single channel, which may lead to some misinterpretation. High speed silicon photodiodes ‘FDS100’ manufactured by Thorlabs were used as photodetectors to convert light signal to an electrical signal (voltage). The photodiode has a wavelength range of 350 to 1100 nm with a rise time of 10 ns. An operational amplifier ‘AD8675’ manufactured by Analog Devices was used to amplify the acquired signal. The photodetectors ‘FDS100’ with an operational amplifier ‘AD8675’ were calibrated manually to adjust the offset. The output signal from the photo-detector gives zero voltage at dark conditions and gives a 5 V signal when incident light falls on the sensor without any interference. Fig. 2a shows an overall schematic representation of the system. The four curvatures are linked with each other to form ‘X’ shape with 90° between adjacent curvatures as viewed along the path of laser beam. Each of these curvatures holds three photodetectors, equally spaced at 15°, as shown in Fig. 2b. The photodetectors on each curvature are numbered 1 through 3, in which detector 1 positioned at 15° corresponds to sample holder, detector 2 positioned at 30°, and detector 3 positioned at 45°

A 16-bit multifunction I/O device PCI-6225 (National Instruments) was used for data acquisition. PCI-6225 interfaced with MATLAB for collecting data from the photodetectors. After preparing a sample, it was placed in the flask holder. The laser beam passes through the sample, and the light was scattered off from the beads. The surrounding photodetectors collected the scattered light with a sampling frequency of 1 kHz. The 12 sensors were positioned at different locations for acquiring data simultaneously and then converted the light intensity into a voltage waveform. The noise from the acquired signal was removed by applying a second-order Butterworth low pass filter with a cutoff frequency of 5 Hz [25]. After assembling the prototype, the data acquisition system was also calibrated to ensure zero
waveform signal when DI water was used as blank sample. The overall system covered with black metallic box to prevent any external noise or unwanted signal. The inner surface of the cover is also black to avoid the reflection or light glare from the side walls.

III. OPTIMUM CONDITIONS

Different tests were performed to estimate three different optimum conditions for attaining higher classification accuracy.

A. ENRICHMENT TIME

The enrichment time is the optimum time period required by HBsAg to combine with IM beads. Although a longer time period may give more prominent results but an optimum time reduces the experimental time. Initially, the sample of IM beads ($1.6 \times 10^4$ ng/mL) was washed twice with PBS to remove impurities and then resuspended in distilled water at 25 °C with final concentration of $3.2 \times 10^2$ ng/mL. IM beads of 0.2 mL were added to 1.5 mL centrifuge tubes containing HBsAg ($1.6 \times 10^3$ ng/mL). The mixed sample was then incubated for 10, 20, 30, 40, 50, and 60 minutes at room temperature. After magnetic separation, the leftover liquid of the whole sample was poured out, and the remaining sample HBsAg enriched IM beads were mixed with 10 mL DI water. The final sample was then incubated for 30 minutes at room temperature. After incubation and magnetic separation, the sample was mixed with DI water to prepare a sample of 10 mL. Each sample was then placed in the chamber of prototype to find the best ratio based on maximum classification accuracy.

B. HBsAg CONCENTRATION

To increase the efficiency of experimental results, the optimum ratio of HBsAg as compared with IM beads was estimated for immunomagnetic separation. The coupling of IM beads and HBsAg were studied at different proportions of 1/1.25, 1/2.5, 1/3.125, 1/3.75, and 1/4.375. IM beads ($3.2 \times 10^2$ ng/mL) of 0.2 mL were washed twice with PBS to remove any contamination. The concentration of IM beads was kept fixed, while the concentration of HBsAg varied in each step accordingly. The mixed sample was then incubated for 30 minutes at room temperature. After incubation and magnetic separation, the sample was mixed with DI water to prepare a sample of 10 mL. Each sample was then placed in the chamber of prototype to find the best ratio based on maximum classification accuracy.

C. WORKING CONCENTRATIONS OF IM-BEADS

For a particular sample of HBsAg, a specific quantity of IM beads is required for performing an identification procedure. The much higher number of IM beads beyond a limit may lead to blank IM beads or without HBsAg enrichment, which may produce unwanted results. To avoid this, a specified working concentration of IM beads must be known for getting better results.

Initially, the suspension of IM beads ($1.6 \times 10^4$ ng/mL) was washed twice with PBS to remove impurities and then resuspended in distilled water at room temperature. The different volumes of IM beads (0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mL) were mixed with DI water, so the volume of final sample was 10 mL. The final samples of IM-beads without HBsAg enrichment were used to find the scattering of light.

Similarly, after washing the IM-beads ($1.6 \times 10^4$ ng/mL), volumes of 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mL were separately added into six 1.5 mL centrifuge tubes containing HBsAg ($2 \times 10^4$ ng/mL) of 20, 40, 60, 80, 100 and 120 µL, respectively. The difference in volume of IM beads and HBsAg for enrichment is due to the results achieved from the ratio of IM beads to HBsAg. The samples were incubated at room temperature for 30 minutes and then used for finding the laser light scattering peaks. The concentration giving a higher number of distinct peaks will be selected for further classification experiments.

IV. ALGORITHM DEVELOPMENT

The algorithm for classifying data consists of two parts: features extraction and Support Vector Machine (SVM). The acquired signal is the time-dependent fluctuations due to scattered light intensity. Twelve different photodetectors (channels) simultaneously collect data. The set of important features were extracted from the data, and then SVM classification was applied to the extracted features for classifying samples.
A. FEATURES EXTRACTION

The feature extraction extracts useful information from the acquired waveform and removes the unwanted information and interferences. Many time domain features extraction methods have been developed for the classification of signals. Rami et al. proposed a features extraction method for myoelectric pattern recognition based on power spectrum characteristics [26]. The proposed work adopts the same technique with some modification to acquire the power spectral features of the waveform. The spectral power behavior of the acquired data for HBsAg identification based on IM beads gives useful information about the signal.

The variation in the signal provides useful information about the waveform for classification. The acquired signals were segmented into small windows, and the features were extracted from each segment. The segment or window created during feature extraction is known as the analysis window, and the size of each window was 250 ms. A power spectral algorithm was applied to each analysis window for feature extraction. The analysis window moves over the waveform data with an increment of 150 ms to find features in each channel. Five different power spectral features were extracted to get the relevant information about the signal behavior. Different waveforms show unique characteristics or power spectral features. The given features depict the variation in the response of signal and help to distinguish waveforms. These features were used further in SVM classification algorithm to classify data.

The acquired time domain signal is represented by \( x[j] \), where \( j \) represents the length \( N \) with the sampling frequency \( f_s \). The frequency-domain signal can represent the information based on the light scattering signal as \( X[k] \) via the discrete Fourier transform (DFT). Parseval’s theorem states that the sum of the square of the function is equal to the sum of the square of its transform. The given theorem is used for derivation of features extraction using a power spectrum [27]:

\[
\sum_{j=0}^{N-1} |x[j]|^2 = \frac{1}{N} \sum_{k=0}^{N-1} |X[k]X^*[k]| = \sum_{k=0}^{N-1} P[k] \tag{1}
\]

Multiplication of \( X[k] \) used its conjugate \( X^*[k] \). The product was divided by \( N \) to give the phase-excluded power spectrum \( P[k] \) where \( k \) is the frequency index value. Due to the symmetrical behavior of the Fourier transform, all positive and negative frequencies were included to deal with the entire spectrum because there is no other method for accessing the power spectrum using a time-domain signal. Therefore, all odd moments will become zero by applying this statistical approach to frequency distribution. According to the definition of a moment \( m \) of the \( n \)th order of power spectral density, \( P[k] \) is given by:

\[
m_n = \sum_{k=0}^{N-1} k^n P[k] \tag{2}
\]

Refer to (2), we can get Parseval’s theorem when \( n = 0 \); the time differentiation property of the Fourier transform is used for other values of \( n \). The \( n \)th derivative of the discrete-time signal is equivalent to multiplication of spectrum with \( k \) raised to the \( n \)th power:

\[
F \left[ \Delta^n x[j] \right] = k^n X[k] \tag{3}
\]

1) ZERO-ORDER MOMENT

The total power of the frequency domain expressed by the features acquired from the zero-order moment:

\[
\tilde{m}_0 = \sqrt{\frac{1}{N} \sum_{j=0}^{N-1} x[j]^2} \tag{4}
\]

2) SECOND AND FOURTH-ORDER MOMENT

The second-order moment can be considered as power according to Hjorth, and its modified spectrum is expressed as \( k^2 P[k] \), which is related to the frequency function \( kX[k] \) [21]:

\[
\tilde{m}_2 = \sqrt{\frac{1}{N} \sum_{j=0}^{N-1} k^2 P[k]} = \frac{1}{N} \sum_{j=0}^{N-1} (\Delta x[j])^2 \tag{5}
\]

Similarly, the root squared fourth-order moment is given by:

\[
\tilde{m}_4 = \sqrt{\frac{1}{N} \sum_{j=0}^{N-1} k^4 P[k]} = \frac{1}{N} \sum_{j=0}^{N-1} (\Delta^2 x[j])^2 \tag{6}
\]

The acquired features were normalized to increase the signal-to-noise ratio; the power transformation applied to \( \tilde{m}_0, \tilde{m}_2, \) and \( \tilde{m}_4 \). The power transformation is done as follows where \( \lambda \) is empirically set to 0.1:

\[
m_0 = \frac{m_0^2}{\lambda}, \quad m_2 = \frac{m_2}{\lambda}, \quad m_4 = \frac{m_4}{\lambda} \tag{7}
\]

First, three extracted features are defined as:

\[
f_1 = \log (m_0) \tag{8}
\]

\[
f_2 = \log (m_0 - m_2) \tag{9}
\]

\[
f_3 = \log (m_0 - m_4) \tag{10}
\]

3) SPARSENESS

It defines the energy of a specific vector packed into only a few components and stated as:

\[
f_4 = \log \left( \frac{m_0}{\sqrt{m_0 - m_2} \sqrt{m_0 - m_4}} \right) \tag{11}
\]

4) WAVEFORM LENGTH RATIO (WL)

WL defines the waveform length feature as the summation of the absolute value of the derivative of the signals:

\[
f_5 = \log \left( \frac{\sum_{j=0}^{N-1} |\Delta^2 x|}{\sum_{j=0}^{N-1} |\Delta^4 x|} \right) \tag{12}
\]

Overall, five different features were extracted from each acquired signal, \( f = [f_1, f_2, f_3, f_4, f_5] \). The data were collected from 12 different channels with a sampling rate of 1000 Hz.
for the identification of HBsAg based on IM separation. Five different features were extracted from each channel resulting in a total of 60 features obtained from 12 channels.

**B. SUPPORT VECTOR MACHINE**

SVM based algorithms are especially promising for the classification of features among various machine learning tools. In this study, SVM was adopted as the machine learning classifier by using the LIBSVM algorithm package [28]. LIBSVM provided four basic kernel functions: linear, polynomial, radial basis function, and sigmoid. We chose the sigmoid as the kernel function because it has a better boundary response and could reflect the distribution of the dataset more accurately. The grid search was used for tuning the parameters of SVM sigmoid kernel. The regularization parameter (C) tuned to 100 and gamma parameter (γ) tuned to 0.01 for getting higher classification accuracy. SVM algorithm allows for non-linear decision boundaries in the parameter space and able to construct decision hyperplanes in a multidimensional space that separate cases of different labels. Supervised SVM learning needs labeled training samples to learn model which enables the classifier to predict the class of unseen data [29].

The measured scattered light features acquired from IM beads and IM beads enriched with HBsAg at twelve different angles labeled as two distinct classes. The data acquired from IM beads were labeled by ‘0’, defined as one class, and the data from IM beads enriched with HBsAg were labeled by ‘1’, defined as another class.

The procedure and methodology for features extraction are the same for training and testing of an SVM classifier. To train a classifier, the data were collected from a known sample of IM beads as well as IM beads enriched with HBsAg. The sixty different experiments were performed for each type of sample with a time duration of 5 minutes. A new sample was prepared for each test every time. The features extraction algorithm extracts five different features from twelve signals and creates a multidimensional data of sixty features. The features of both classes were arranged randomly, and separated into training data and testing data to evaluate the learned model. The SVM classifier was trained to determine the hyper-plane for separating the data of distinct features. In testing phase, the testing dataset were used to evaluate the classification accuracy. The overall flowchart for the classification of HBsAg based on immunomagnetic separation and SVM is shown in Fig. 3.

**C. PERFORMANCE ANALYSIS**

To obtain an optimal utilization of all information in the training data, the trained model was evaluated by cross-validation. Cross-validation is a technique for determining the classification results on other independent sets of data. The cross-validation splits the dataset into a defined number of groups S. The dataset of S-1 groups were applied for training a model and the remaining one was used for validation. This approach was repeated for all possible choices of evaluation groups. The performance evaluated from the average of all results. The confusion matrix resulting from the cross-validation was subsequently used to calculate the other performance metrics.

We next calculated true positive (\(T_P\)), true negative (\(T_N\)), false positive (\(F_P\)), and false negative (\(F_N\)), where \(T_P\) and \(T_N\) represent the correct classification of the presence and absence of HBsAg data. \(F_P\) and \(F_N\) represent the misclassification of the presence and absence of HBsAg data. Based on these \(T_P\), \(T_N\), \(F_P\), \(F_N\) parameters, a confusion matrix was plotted to analyze the performance of an algorithm statistically. The different performance metrics for the sample were calculated as accuracy, precision, recall, specificity, and F-score, where the accuracy is the number of all correctly predicted samples to a total number of events. Precision is the correctly predicted positive observations to all observations in the actual class. Recall measures the proportion of actual positives that were correctly identified, and specificity measures the proportion of actual negatives that were correctly identified. The F-score is defined as the weighted harmonic mean of precision and recall of the classification test [30].
Using samples with a known concentration of 50,616.61 IU/mL, one of the known HBsAg positive HBV carrier serum to find the limit of detection. The Sample A system and SVM. 10 healthy control subjects were analyzed by laser scattering 10 patients known to be HBsAg-positive HBV carriers and samples of healthy control subjects. Serum samples from chronic hepatitis B patients, and negative results for 10 serum obtained results were positive for the 10 serum samples of control. All of the 20 serums were tested by using CLIA. The 20 different clinical serums were obtained from the Gulou Hospital, Nanjing, China, to validate the developed system and methodology. All specimens were separated from clots and stored at −80°C. Out of 20 serum specimens, the 10 samples were obtained from the patients chronically infected with HBV, and the remaining 10 samples were collected from healthy control subjects known as a negative control. All of the 20 serums were tested by using CLIA. The obtained results were positive for the 10 serum samples of chronic hepatitis B patients, and negative results for 10 serum samples of healthy control subjects. Serum samples from 10 patients known to be HBsAg-positive HBV carriers and 10 healthy control subjects were analyzed by laser scattering system and SVM, Serial dilution tests were conducted on HBsAg-positive serums to find the limit of detection. The ‘Sample A’ was one of the known HBsAg positive HBV carrier serum samples with a known concentration of 50,616.61 IU/mL. Using ‘Sample A’, a total of 10 different diluted samples were prepared with a dilution factor of 1:1 for estimating the limit of detection for the proposed method. The prepared diluted samples were then used for the HBsAg enrichment using IM beads. The IM beads (1.6 × 10⁴ ng/mL) were used for the immunomagnetic separation of HBsAg from the diluted serum. After immunomagnetic separation, the acquired data from the serum samples by using the laser light scattering were used for the HBsAg identification. The procedure for immunomagnetic separation and sample preparation for obtaining data by using laser scattering is the same as discussed previously (Section 2.1). The limit of detection for HBsAg identification was evaluated by using the acquired classification accuracies of all the diluted samples. Then, all of the remaining clinical serum samples were used for performing the HBsAg identification test using immunomagnetic separation and measuring the scattered light. The acquired data were used further to find the power spectral features, and the SVM classifier classifies the features as positive or negative for HBsAg test.

The overall classification accuracy (%) is defined as:

\[
\text{Accuracy} = \frac{TP + TN}{Total \ number \ of \ dataset} \times 100
\]

(13)

The performance metrics can be defined in term of given parameters:

\[
\text{Accuracy} = \frac{TP + TN}{TP + TN + FP + FN}
\]

(14)

\[
\text{Precision} = \frac{TP}{TP + FP}
\]

(15)

\[
\text{Recall} = \frac{TP}{TP + FN}
\]

(16)

\[
\text{Specificity} = \frac{TN}{FP + TN}
\]

(17)

\[
F - \text{score} = \frac{2 \times TP}{2 \times TP + FP + FN}
\]

(18)

V. SYSTEM VALIDATION BASED ON CLINICAL SAMPLES

The 20 different clinical serums were obtained from the Gulou Hospital, Nanjing, China, to validate the developed system and methodology. All specimens were separated from clots and stored at −80°C. Out of 20 serum specimens, the 10 samples were obtained from the patients chronically infected with HBV, and the remaining 10 samples were collected from healthy control subjects known as a negative control. All of the 20 serums were tested by using CLIA. The obtained results were positive for the 10 serum samples of chronic hepatitis B patients, and negative results for 10 serum samples of healthy control subjects. Serum samples from 10 patients known to be HBsAg-positive HBV carriers and 10 healthy control subjects were analyzed by laser scattering system and SVM. Serial dilution tests were conducted on HBsAg-positive serums to find the limit of detection. The ‘Sample A’ was one of the known HBsAg positive HBV carrier serum samples with a known concentration of 50,616.61 IU/mL. Using ‘Sample A’, a total of 10 different diluted samples were prepared with a dilution factor of 1:1 for estimating the limit of detection for the proposed method. The prepared diluted samples were then used for the HBsAg enrichment using IM beads. The IM beads (1.6 × 10⁴ ng/mL) were used for the immunomagnetic separation of HBsAg from the diluted serum. After immunomagnetic separation, the acquired data from the serum samples by using the laser light scattering were used for the HBsAg identification. The procedure for immunomagnetic separation and sample preparation for obtaining data by using laser scattering is the same as discussed previously (Section 2.1). The limit of detection for HBsAg identification was evaluated by using the acquired classification accuracies of all the diluted samples. Then, all of the remaining clinical serum samples were used for performing the HBsAg identification test using immunomagnetic separation and measuring the scattered light. The acquired data were used further to find the power spectral features, and the SVM classifier classifies the features as positive or negative for HBsAg test.

The trained SVM classifier was created by using the acquired features from known samples. The features obtained from the IM beads sample enriched with HBsAg were labeled as ‘1’, and the remaining features acquired from the IM-beads sample without HBsAg were labeled as ‘0’. Therefore, the classification results of the acquired features from the test sample will be presented by labels, i.e., ‘1’ (positive) and ‘0’ (negative). The percentage identification accuracy evaluated by using the number of positive labels to the total number of labels obtained from the test sample. The identification accuracy of the test sample is given by (13). For testing each serum sample, the identification test was performed three times to get mean identification accuracy and standard deviation. Based on the acquired results, the threshold value was selected to classify the sample response to be positive or negative. Therefore, the blind validation experiments were performed by setting different percentage values as limit or threshold value for HBsAg positive detection, to validate the practicability and feasibility of the method. If the obtained percentage accuracy is above the threshold, the testing sample will be considered as positive for HBsAg. Similarly, the percentage accuracy below threshold will be considered as negative HBsAg. Five different groups were created with different numbers of positive and negative samples. The threshold value selection is based on achieving higher classification accuracy for all samples.

VI. RESULTS AND DISCUSSION
A. SAMPLE PREPARATION

The two different samples were used for immunomagnetic separation of HBsAg: one sample contains HBsAg while the other without HBsAg. Both samples were used to take SEM images to analyze the characteristics of particles. ZEISS Ultra Plus Scanning Electron Microscope having a resolution of 1 nm at 15 kV was used to take SEM images. Fig. 4 shows the SEM images of IM beads after
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FIGURE 5. (a) Internal view of the system, (b) Arrangements of twelve photodetectors in forward direction of sample, and (c) External cover of the system to avoid external light (dimension: 50 cm × 30 cm × 30 cm).

immunomagnetic separation and shows that IM beads enriched with HBsAg have different surface characteristics as compared to blank IM beads. The sample was then analyzed with a light scattering instrument. The system further classifies the blank IM beads and the IM beads enriched with HBsAg via laser light scattering and SVM.

B. DEVELOPMENT OF PROTOTYPE

A prototype was successfully developed to identify the presence of HBsAg on IM beads. Fig. 5a shows the internal structure and different components of the device. The location of the sensors was adjusted according to the Mie scattering theory [31], [32]. Fig. 5b shows the arrangement of twelve sensors at various positions, and Fig. 5c shows the system covered by a black box to avoid any external noise or interference.

C. OPTIMUM CONDITIONS FOR DATA ACQUISITION

During the identification process, IM beads were mixed with the test sample, and if the target sample contains HBsAg, then it would be captured by the IM beads. The optimized conditions and parameters significantly affect the classification accuracy.

The ability of HBsAg to bind with anti-HBsAg-coated paramagnetic microparticles depends on the incubation time period. The different samples of IM beads (3.2 × 10^2 ng/mL) were mixed with HBsAg (8 × 10^2 ng/mL) in parallel and incubated for different timings. After magnetic separation, the samples were used to find classification accuracy. The higher classification accuracy achieved at a time duration of 30 minutes for the enrichment of HBsAg with IM beads (Fig. 6a). The graph shows that the accuracy of the classifier remains higher after 30 minutes. Therefore 30 minutes is the optimum time duration for enrichment of HBsAg with IM beads.

Immunomagnetic separation of HBsAg is a key process in HBsAg detection system. The appropriate ratio of IM beads to HBsAg was tested to optimize the IM beads and HBsAg interaction. Different tests for known ratios were performed to evaluate the identification accuracy of HBsAg. The optimum ratio for binding of IM beads and HBsAg sample was estimated to be 1/2.5 because this led to higher accuracy of 80% (Fig. 6b). The lower concentration of HBsAg leads to less binding and some of the beads would be without HBsAg linking. This causes the false positive prediction of the sample and greatly affects the classification accuracy. Only a specific proportion of HBsAg can be combined with IM beads. At higher concentration or volume of HBsAg as compared with IM beads, will concentrate the sample with HBsAg. The given problem may lead to the change in scattering of light and the graph may show lower classification accuracy. Therefore, the minimum concentration ratio of IM beads to HBsAg must be 1/2.5.

The power spectral features from the acquired waveform depend on the variation of scattered light intensity and play a key role in classification. The change in the concentration of sample gives the variation in output signal. When concentration is too low, the scattering from the sample is weak. Similarly, when the concentration is very high, the measurement results will be distorted due to particle-particle interaction. In either case, the output results will be distorted. The number of peaks represents the variations in data due to higher laser light scattering. The different volumes of IM beads (1.6 × 10^4 ng/mL) mixed with DI water to make a suspension of 10 mL for finding laser light scattering. The different samples were tested to acquire the maximum number of peaks: Fig. 6c shows that the number of peaks was low at lower concentration and then it increases gradually. The IM beads with a volume of 0.2 mL (3.2 × 10^2 ng/mL) give the higher number of peaks. Similarly, the IM beads enriched with HBsAg also gives the higher number of peaks at a concentration of 3.2 × 10^2 ng/mL. When the concentration of sample is too high, the number of peaks drop to lower value. Therefore, it is important to keep particle concentration within a specific limit during experiments.

D. PERFORMANCE EVALUATION AND ANALYSIS

Data acquired from the samples of blank IM beads and HBsAg enriched IM beads to evaluate the performance of laser scattering system. The sixty different experiments for each of the two samples were tested separately under optimized conditions to acquire data of 5 minutes in each experiment. Therefore, a total of 120 samples of both classes were used for collecting data. Fig. 7 shows the data from
IM beads and IM beads enriched with HBsAg. The plots have a distinctive signal and peak intensity between 20 and 25 s. Different colors represent different channels. Each data has distinct peaks at different time period.

Khushaba et al. [33] proposed a method for feature extraction for myoelectric pattern recognition based on power spectrum characteristics. The spectral power behavior of the acquired data from the IM beads for HBsAg identification showed a unique signal transition in different experiments. Therefore, feature extraction methods were used for the classification of HBsAg based on IM beads. The five different power spectral features were extracted from the acquired twelve waveforms of each sample (total of 60 features). The correlation analysis was performed for individual features by using a $12 \times 12$ heat-map matrix. The heat-map presents an overview of the visual correlation between different features, as shown in Fig. 8 (a-e). The correlation analysis signifies the relationship between two features. The value of correlation coefficient exists between $-1$ to $+1$. If the two features are completely matching, then the correlation coefficient is $+1$. If the relationship between two features is opposite, the correlation coefficient is $-1$. The correlation coefficient equals to 0 means that no relationship exists between features. In Fig. 8 (a-e), the given correlation analysis depicts that there is much variation exist between all of the five power spectral features acquired from twelve photo-detectors. Therefore, using all of the 60 features will not tend to reduce the overall classification results.

The SVM classification was used to evaluate the discrimination performance of the prototype for the identification of HBsAg. For the classification of non-linearly separable features, SVM algorithms create a non-linear decision boundary in the parameter space [34]. The training data was provided to a learning system that develops a classifier model for predicting the class of testing data based on the training data [35], [36]. The classification result of the trained model was evaluated by a 10-fold cross-validation. The confusion matrix built from the resulting cross-validation was used to analyze the performance of the classifier.

Fig. 9 shows the confusion matrix based on the features of two different samples. Each row of the matrix represents the actual class of sample data, while each column represents the predicted samples classified by SVM. The detailed description is given here to better understand about given
The performance assessment of the proposed methodology is carried out by computing the parameters, i.e., $T_P$, $T_N$, $F_P$, $F_N$ based on Fig. 9. The mean values of accuracy, precision, sensitivity, specificity, and F-score were evaluated based on (13-18), and presented in Table. 1. The classification accuracy attained by SVM classifier for the identification of HBsAg is 87.76%. Fig. 10 shows the box plot of five different classification parameters that were obtained from each of the 10-fold cross-validations of the dataset. The box-plot depicts that there is much less variation in the performance measures of different partitions of data with lower standard derivation. The given results validate the proposed approach for the identification of HBsAg based on immunomagnetic separation and laser light scattering with SVM.

### E. System Validation Based on Clinical Samples

The 'Sample A' with an actual concentration of 50616.61 IU/mL was diluted with 1:1 to prepare 10 different
diluted samples for testing the limit of detection. The quantitative detection range was 98.86 to 3163.5 IU/mL.

The HBsAg identification test using laser scattering was performed on all of the 20 clinical samples. The features acquired from the sample were tested by the trained model. The SVM classifier classifies the features in terms of HBsAg positive (labelled by 1) or HBsAg negative (labelled by 0). The prediction of the sample is given by percentage evaluation of positive outcomes. Fig. 11(a) shows the results for HBsAg positive serum samples, presenting the classification accuracy in terms of mean identification value and standard derivation. Each of the test was repeated five times to obtain the standard derivation in final results. The acquired results for different HBsAg positive serums showed different values, depending on the number of features classified by the SVM classifier. The minimum mean percentage classification value acquired was 76.3%, and the maximum value of 97.07%. The results obtained from 10 healthy control subjects showed a negative response, as shown in Fig. 11(b). A total of three out of ten HBsAg negative samples give small values for positive classification, and the remaining samples show zero value.

The maximum percentage value obtained for negative control samples was 22%, which shows a marked difference between the obtained classification results of positive control samples and negative control samples.

The blind test validation was performed to attain the results by setting a threshold value of percentage accuracy for HBsAg detection. The threshold value was assigned as a limiting value for classifying a positive sample. Below the threshold value, the sample will be classified as negative. Different groups of samples for both positive and negative control of HBsAg were randomly selected, and various threshold values were applied to attain the results. The classification accuracy of the testing sample above a threshold value will be considered as HBsAg positive, while the samples having a classification accuracy below the threshold will be considered as HBsAg negative. The optimized threshold value was selected by performing blind tests, and the acquired results were compared with the known results of CLIA. The obtained results in Table. 2 shows that increasing the threshold value

| No. of Samples | CLIA Positive/ Negative | 75% Positive/ Negative | 80% Positive/ Negative | 85% Positive/ Negative | 90% Positive/ Negative |
|----------------|-------------------------|------------------------|------------------------|------------------------|------------------------|
| 10             | 5/5                     | 5/5 (100%)             | 4/6 (90%)              | 4/6 (90%)              | 4/6 (90%)              |
| 13             | 7/6                     | 7/6 (100%)             | 6/7 (92.3%)            | 5/8 (84.6%)            | 3/10 (69.2%)           |
| 15             | 9/6                     | 9/6 (100%)             | 8/7 (93.3%)            | 7/8 (86.6%)            | 5/10 (73.3%)           |
| 17             | 8/7                     | 8/7 (100%)             | 7/8 (93.3%)            | 6/9 (86.6%)            | 4/11 (73.3%)           |
| 19             | 10/9                    | 10/9 (100%)            | 9/10 (90.7%)           | 8/11 (89.4%)           | 6/13 (78.9%)           |

The mean percentage classification: (a) Ten different HBsAg positive serum samples, and (b) Ten different HBsAg negative serum samples.
increases the sensitivity but it also reduces the accuracy of the algorithm. Therefore, the optimum percentage classification for the HBsAg detection estimated to be 75% for attaining 100% accuracy for the given serum samples when compared with the known results from CLIA. The overall results for HBsAg detection from the clinical serum samples were matching with those of conventional Chemiluminescence Immunoassay (CLIA).

VII. CONCLUSION

The main goal of this paper was to develop a new system and validate the method for the identification of HBsAg utilizing IM beads coated with polyclonal anti-HBsAg. The immunomagnetic separation method used here can theoretically isolate HBsAg because it is based on the magnetic separation of HBsAg. SVM is a popular supervised classification method for both linear and non-linear data, and widely applied in many applications of biomedical engineering. The light scattering data was used to train the SVM classifier. The same methodology was used on test samples for detection purpose. This method achieves a higher classification accuracy of 87.76% and is technically simple.

The proposed method is rapid and does not require complex sample preparation as compared with other methods. One of the drawbacks of the iPCR test is false-positive results due to carryover contamination of previously amplified products and false-negative results due to the presence of amplification inhibitors in clinical samples. CLIA and ELISA are very expensive and time-consuming and difficult to use. In contrast, the proposed method is simple and does not require a skilled operator. Moreover, the experiment required less than 2 hours. Thus, the proposed approach not only provides a novel method for the detection of HBV, even in serum but also overcomes some of the drawbacks of conventional methods (ELISA), such as the extended testing time and the multiple washing steps. The 20 different clinical serum samples, including 10 HBsAg positive serum samples, and the remaining 10 healthy control subjects were used to test the given method for HBsAg detection. The blind test was performed to validate the system, and it shows 100% accuracy at a threshold value of 75%. The results of the blind study increases the sensitivity but it also reduces the accuracy of the algorithm. Therefore, the optimum percentage classification for the HBsAg detection estimated to be 75% for attaining 100% accuracy for the given serum samples when compared with the known results from CLIA. The overall results for HBsAg detection from the clinical serum samples were matching with those of conventional Chemiluminescence Immunoassay (CLIA).

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