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RESEARCH ARTICLE

Highly Sensitive Hepatitis B Virus Identification by Antibody-Aptamer Sandwich Enzyme-Linked Immunosorbent Assay

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Abstract:

Hepatitis B virus (HBV) infection is considered a major global health problem, causing various health issues, including cirrhosis, hepatitis, and liver cancer. The risk of developing such complications increases when the viral load is above 10^5 copies/mL. Early identification of HBV infection is imperative to preventing the spread of infection to other parts of the body. Although various sensing methods have been developed to identify HBV, researchers are still working toward developing cheap, easy, and sensitive detection methods. We developed a highly sensitive and rapid HBV detection method using nanomaterials on enzyme-linked immunosorbent assay (ELISA). Aptamer-antibody was utilized as the detection probe and immobilized on a zeolite-modified ELISA plate to detect the HBV biomarker hepatitis B surface antigen (HBsAg). To enhance the detection of HBsAg, aptamer and antibody were attached to gold nanoparticle through electrostatic interaction and immobilized on the zeolite-modified ELISA plate through amine linker. This probe-modified ELISA plate detected low levels of HBsAg, with a detection limit of 0.1 ng/mL. Furthermore, serum spiked experiments showed increment of absorbance with increasing HBsAg concentration, but control trials with other biomolecules showed no increment of absorbance, showing the specific and selective detection of HBsAg. This nanomaterial-modified ELISA plate can detect low levels of HBsAg and help in the diagnosis of HBV infection in its early stages.

Keywords: Enzyme-linked immunosorbent assay, Hepatitis B surface antigen, Hepatitis B virus, Nanomaterial, Zeolite

1. Introduction

The combination of biotechnology and nanomaterials offers an excellent platform for the successful utilization of surface functionalization in various diagnostic techniques [1,2]. Biosensors have been widely used for diagnosing diseases, drug delivery, monitoring environmental issues, genetic mutation identification, and drug screening. Early identification of diseases is crucial for preventing the spread of viral diseases, which requires highly sensitive, specific, and cheaper methods [3-5]. The improvement of biosensors predominantly relies on two factors: the surface functionalization and the binding affinity of biomolecules. Various researches are working toward improving the surface functionalization of biomolecules through various methods [6], and nanomaterials contribute

to various aspects that improve the functionalization of biomolecules.

Nanomaterials are nanoscale materials that are synthesized in various shapes and are <100 nm in size. Nanomaterials are widely applied in various fields, including the clothing industry, electronics, environmental, and pharmaceuticals. In particular, the use of nanomaterials is inevitable in the medical field, where they are applied in drug delivery, diagnosis, and therapy [7-10]. Since nanosized materials can easily enter the human body through ingestion, inhalation, and also the skin, they can be used for targeting particular cells to treat diseases. Apart from that, nanomaterials are applied in the field of biosensors to enhance the detection of target molecule. Various types of nanomaterials have been successfully applied on sensing substrates to increase the attachment of biomolecules. Here, two nanomaterials, zeolite and gold, were used to attach the aptamer and antibody probe on the enzyme-linked immunosorbent assay (ELISA) polystyrene (PS) plate to detect hepatitis B surface antigen (HBsAg). Zeolite is the composite of silica and alumina, which has a larger size pole, higher surface area, and excellent mechanical and chemical stability. Zeolite consists of $[SiO_4]^{4-}$ and $[AlO_4]^{4-}$, which create a negatively charged surface. Zeolites are synthesized in various shapes and sizes and used for different applications, such as biosensor, catalyst, drug delivery, and heavy metal removal [11]. In recent years, the fascination of zeolite in the field of biosensors is attributed to its high conductivity, high resistance, and tunable redox potential [12-14]. In this research, zeolite was used to attach the aptamer-antibody conjugated gold nanoparticle (GNP) on the ELISA plate. Gold is a versatile metal and can be effectively used in various biosensing applications. Due to its biocompatibility and easy functionalization, biomolecules are conjugated with gold and used to enhance analytical performances. In this work, aptamer-antibody conjugated GNP was attached to the ELISA PS plate to quantify the level of HBsAg.

ELISA is a widely used method that helps screen various diseases in the medical field [15]. Until now, the screening and identification of major diseases have been done through ELISA due to its high sensitivity, without involving hazards, such as radioisotope [16-18]. Most ELISA experiments are based on the interaction of antigen and its specific antibody [19,20]. Improving ELISA is a necessary step to enhance the biomolecular interactions on the ELISA plate [21]. Biomolecule immobilization on the PS plate is challenging, especially for smaller-sized biomolecules, such as peptide, DNA, RNA, and aptamer, all of which are not stable on the ELISA plate. Polymers, nanomaterials, are useful for attaching smaller-sized molecules onto the ELISA plate. Here, zeolite and GNP were used to increase the number of probe molecules on the PS plate.

2. Materials and methods

2.1. Reagents and biomolecules

Human serum, 3-(aminopropyl)triethoxysilane (APTES), secondary antibody-horseradish peroxidase (HRP), and GNP were purchased from Sigma Aldrich, USA. We received bovine serum albumin (BSA) and substrate for HRP from Promega, USA. Both mouse anti-HBsAg antibody and HBsAg were purchased from Shanghai Chao Yan Biotechnology Ltd., China. ELISA plate was ordered from Becton Dickinson, France. Anti-HBsAg aptamer was synthesized and received from a local supplier [22].

2.2. Preparation of gold nanoparticle-aptamer-antibody conjugates

To prepare the GNP-aptamer-antibody conjugates, the surface of GNP was first modified with antibody through the linker 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)-N-hydroxysuccinimide (NHS). For this, 1 mL of GNP was mixed with 5 mM of 16-mercaptoundecanoic acid (MDA) and then rested for 1 h. Subsequently, the bound GNP-16-MDA was separated by centrifugation at a speed of 10,000 rpm for 10 min. The bound GNP-16-MDA was further treated with 1 mM of EDC-NHS and continuously stirred for 1 h at room temperature after mixing 200 nM of anti-HBsAg. The final product (antibody conjugated GNP) was, then, stored in a refrigerator. To attach the aptamer to the GNP-antibody, thiolated anti-HBsAg aptamer was mixed with the solution and rested for 1 h; then, the conjugated GNP-antibody-aptamer (dual probe) was separated by centrifugation.

2.3. Gold nanoparticle-antibody optimization

The GNP-antibody concentration was optimized as follows: (i) ELISA well was treated with 1% of potassium hydroxide (KOH); (ii) zeolite-APTES (1 mg/mL of zeolite with 1% of APTES) was added for 1 h and washed with ethanol; (iii) a GNP-antibody concentration of 0 – 4 mg/mL was added and left for 1 h; (iv) 2% BSA was added and left for 1 h; (v) secondary antibody-HRP (1:1000 dilution) was dropped and rested for 1 h; and (vi) substrate for HRP was added. The absorbance was recorded at 405 nm.

2.4. Gold nanoparticle-antibody-aptamer optimization

GNP-antibody-aptamer conjugation was optimized as follows: (i) ELISA well was treated with 1% of KOH; (ii) zeolite-APTES (1 mg/mL of zeolite with 1% of APTES) was added for 1 h and washed with ethanol; (iii) GNP-antibody-aptamer (optimized antibody with various aptamer concentration) was added and left for 1h; (iv) 2% BSA was added and left for 1h; (v) HBsAg with 50 ng/mL was added; (vi) HBsAg-monoclonal antibody was added and left for 1 h; (vii) secondary antibody-HRP (1:1000 dilution) was dropped and rested for 1 h; and (viii) substrate for HRP substrate was added. The absorbance was recorded at 405 nm.

2.5. Detection of HBsAg with dual probe

HBsAg detection with dual probe was carried out as follows: (i) ELISA well was treated with 1% of KOH; (ii) zeolite-APTES (1 mg/mL of zeolite with 1% of APTES) was added for 1 h and washed with ethanol; (iii) optimized dual probe was added and left for 1 h; (iv) 2% BSA was added and left for 1 h; (v) HBsAg of 0 – 50 ng/mL was added; (vi) HBsAg monoclonal antibody was added and left for 1 h; (vii) secondary antibody-HRP (1:1000 dilution) was dropped and rested for 1 h; and (viii) HRP substrate was added. The absorbance was recorded at 405 nm. Each experiment was conducted 3 times with different ELISA plates. The surface modification and the concentration of biomolecules remained the same. The average was calculated and plotted in a graph.

2.6. Specific and selective detection of HBsAg

For selective experiment, HBsAg (0 – 50 ng/mL) was spiked in human serum and performed the same way as previously described. For specific experiment, four different control experiments were performed: (i) without dual probe; (ii) without secondary antibody; (iii) without HBsAg; and (iv) with control protein hepatitis C core antigen (HCVcAg). All other experimental conditions were followed as described previously.

3. Results and discussion

A single probe of GNP conjugated aptamer and antibody was introduced here to detect the hepatitis B virus (HBV) target HBSAg on the ELISA plate. **Figure 1** is a schematic illustration of HBsAg identification by sandwich ELISA with aptamer and antibody as the probe. As shown in the figure, GNP-antibody-aptamer conjugates were attached to the PS well through the zeolite-APTES linker. On the KOH-treated ELISA well, zeolite-APTES was added, and the dual probe was attached through the amine linker. After that, HBsAg was allowed to interact with immobilized aptamer and antibody and sandwiched with polyclonal HBsAg antibody. Subsequently, secondary antibody-HRP was added, followed by HRP substrate. In general, sandwich ELISA is conducted through aptamer-antibody or antibody-antibody interaction [23-25]. Here, the dual probe of aptamer-antibody conjugated GNP was used as the capture probe to attract more HBsAg. DNA aptamer was selected against HBsAg from the randomized library of molecules using the systematic evolution of ligands by exponential enrichment (SELEX) method. Magnetic nanoparticles were used to separate the bound molecule from each round of SELEX. After 13 rounds, the specific HBsAg aptamer was selected, and the chemiluminescent intensity range of HBsAg with its aptamer was found to be in the range of 1 – 200 ng/mL. The selected aptamer against HBsAg was attached onto the surface of the GNP and used here as a detection probe to detect HBsAg. Along with the aptamer, polyclonal HBsAg antibody was attached onto the uncovered GNP surface and developed as a single probe with aptamer and antibody for the detection of HBsAg. Since aptamers are more sensitive and specifically bind to their targets [26,27], they attract more HBsAg. Apart from that, as more antibodies and aptamers attach onto the GNP, more HBsAg will bind to them.
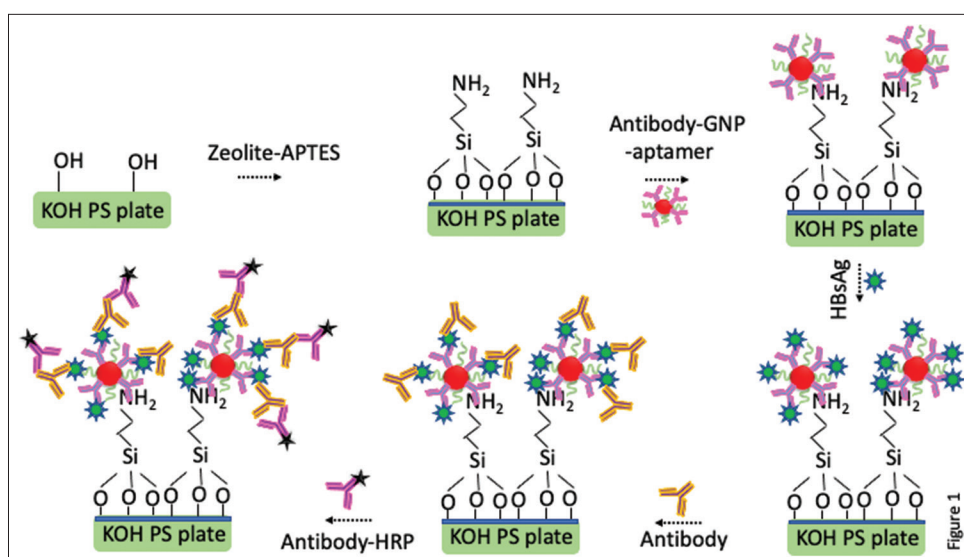
Figure 1. Schematic illustration of HBsAg detection by sandwich ELISA with aptamer and antibody as the probe. GNP-antibody-aptamer conjugates attached onto the ELISA well through the zeolite-APTES amine linker. On the KOH-treated ELISA well, zeolite-APTES was added, and the dual probe was attached through the amine linker. HBsAg was then allowed to interact with immobilized aptamer and antibody and sandwiched with polyclonal HBsAg antibody. Subsequently, secondary antibody-HRP was introduced, and the substrate for HRP was added. APTES: 3-(aminopropyl)triethoxysilane; ELISA: Enzyme-linked immunosorbent assay; GNP: Gold nanoparticle; HBsAg: Hepatitis B surface antigen; HRP: Horseradish peroxidase; KOH: Potassium hydroxide.

3.1. Polyclonal anti-HBsAg antibody optimization

Since the attachment of the probe on the ELISA well plays a key role in detecting HBsAg, it is mandatory to optimize the necessary antibody concentration on the GNP surface. To optimize the antibody concentration, antibody-GNP of 0.5 – 4 mg/mL was attached on the ELISA well and detected by secondary antibody-HRP. As shown in **Figure 2A**, with zero antibody, the absorbance was 0.04 OD, but the absorbance increased to 0.185 OD with an antibody concentration of 0.5 mg/mL. Further increasing the antibody concentration to 1, 2, 3, and 4 mg/mL, the absorbance was noted to be 0.23, 0.49, 0.584, and 0.585 OD, respectively. From the result, we identified that the OD was saturated from a concentration of 3 mg/mL of anti-HBsAg antibody; hence, for further experiments, 3 mg/mL of antibody was used.

3.2. Dual probe optimization

After optimizing the antibody concentration, it is mandatory to optimize the aptamer concentration to cove the excess surface of the GNP. For this, 0.5–3 μM of aptamer was attached on the surface of the antibody-modified GNP, placed on the

amine-modified ELISA well, and detected by HBsAg. As shown in **Figure 2B**, with zero aptamer, the absorbance was recorded as 0.434 OD, but when the aptamer was increased to 0.5 μM, the absorbance increased to 0.35 OD, thus confirming the binding of HBsAg with aptamer and antibody. Further increasing the aptamer concentration to 1, 2, and 3 μM, the absorbance increased to 0.535, 0.586, and 0.59 OD, respectively. It was clearly seen that the absorbance was saturated from 2 μM of aptamer; thus, this concentration was used for further experiments.

3.3. Sandwich detection of HBsAg with dual probe and antibody

HBsAg was detected by sandwich assay on ELISA plate. A HBsAg concentration of 0 – 50 ng/mL was detected. The conventional sandwich ELISA and our modified ELISA were compared. As shown in **Figure 3A**, with a HBsAg concentration of 0.1 ng/mL, the absorbance did not increase with the conventional essay, but it increased to 0.1 OD with the modified ELISA. Further increasing the concentration of HBsAg, higher absorbance was noted in all concentrations of HBsAg. As shown in
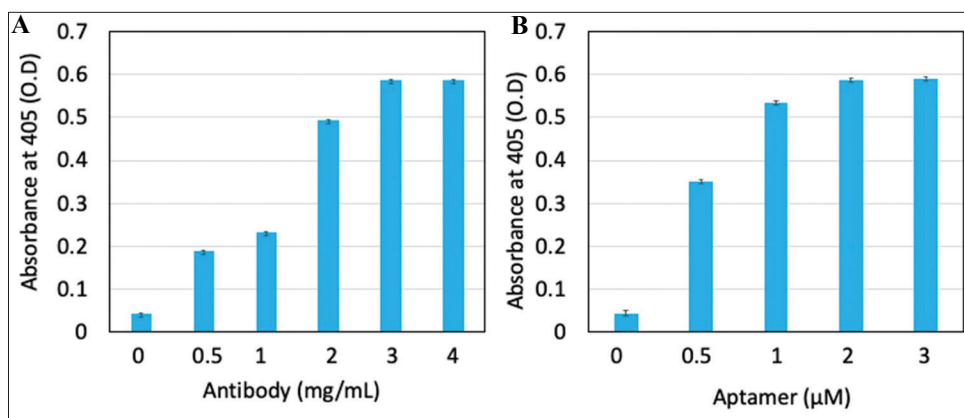
Figure 2. (A) Polyclonal anti-HBsAg antibody optimization. Antibody-GNP of 0.5 – 4 mg/mL was attached on the ELISA well and detected by secondary antibody-HRP. The absorbance increased with an increase in antibody concentration, and it was saturated from a concentration of 3 mg/mL of antibody. (B) Aptamer optimization. Aptamers with a concentration of 0.5 – 3 μM were attached onto the surface of antibody-modified GNP, placed on the amine-modified ELISA well, and detected by HBsAg. The absorbance increased with an increase in antibody concentration, and it was saturated from 2 μM of aptamer. ELISA: Enzyme-linked immunosorbent assay; GNP: Gold nanoparticle; HBsAg: Hepatitis B surface antigen.
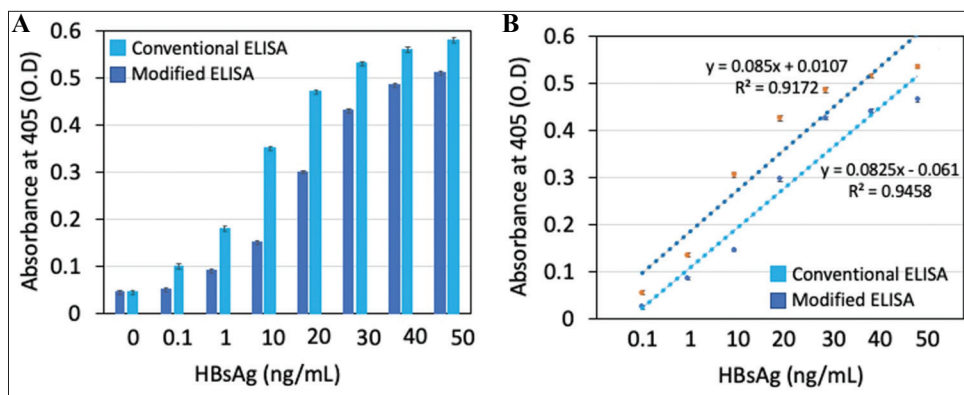
Figure 3. (A) Sandwich detection of HBsAg with dual probe and antibody. The conventional sandwich ELISA and our modified ELISA were compared. Compared with conventional ELISA, the absorbance increased in all concentrations of HBsAg with the modified ELISA. (B) The difference in absorbance was calculated and plotted in an Excel sheet, and the limit of detection of HBsAg was 10 ng/mL with conventional ELISA and 0.1 ng/mL with the modified ELISA. ELISA: Enzyme-linked immunosorbent assay; HBsAg: Hepatitis B surface antigen.

Figure 3A, with 0.1, 10. 20, 30, 40, and 50 ng/mL HBsAg, the OD was 0.05, 0.019, 0.15, 0.3, 0.43, 0.49, and 0.51 OD, respectively, with conventional ELISA. However, the OD was almost double at the lowest concentration of HBsAg with the modified ELISA. At the highest concentration, a smaller increment was noted due to the saturation. The absorbance improved with the modified ELISA compared with the conventional ELISA due to the attraction of a greater number of HBsAg with the GNP conjugated aptamer and antibody. The difference of absorbance was calculated and plotted in an Excel sheet, and the limit of detection of

HBsAg was found to be 10ng/mL with conventional ELISA and 0.1 ng/mL with the modified ELISA (**Figure 3B**). In any biosensing technique, probe immobilization plays a major role in improving sensitivity. Apart from that, only one type of probe molecule is often used to identify the target molecule in conventional ELISA. In this modified ELISA, we used two probes, aptamer and antibody, and attached them on the GNP surface. This helps attach a higher number of detection probe of aptamer and antibody on the ELISA well and enhance the sensitivity. Compared with conventional ELISA, the OD increased in all concentrations of HBsAg
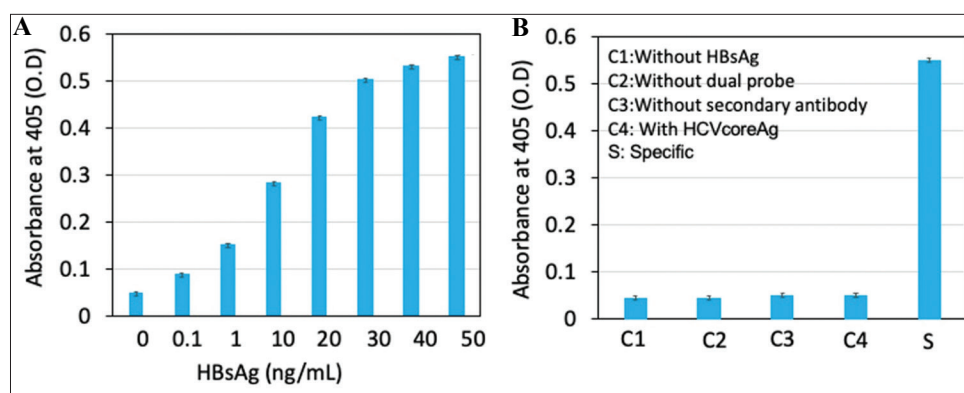
Figure 4. (A) Selective detection of HBsAg (0 – 50 ng/mL) was conducted by spiking HBsAg in human serum. The absorbance increased with an increase in HBsAg concentration, indicating the selective detection of HBsAg. (B) Specific experiment was conducted with four control experiments: (i) without dual probe; (ii) without secondary antibody; (iii) without HBsAg; and (iv) with control HCVcAg. The absorbance level did not increase in the control experiments, but a clear increment of absorbance was recorded in the specific experiment. HBsAg: Hepatitis B surface antigen; HCVcAg: Hepatitis C virus core antigen.

and reached a detection limit of 0.1 ng/mL, which is 10 times lower than the conventional ELISA (1 ng/mL). HBV associated antibody/antigen detection is 10- to 100-fold lower than with other sensing systems using nucleic acid-based identification methods [28,29]. There are a limited number of comparative tests between the novel biosensor and the PCR-based detection system. The sensitivity of the sensor system is known to be 10^3 copies/mL. In terms of conventional ELISA, the detection limit of HBsAg is approximately 0.5 ng/mL [30]. Our sensing system, which utilizes a single probe of aptamer and antibody on GNP, enhances the analytical performances and improves the sensitivity to 0.1 ng/mL.

3.4. Specific and selective detection of HBsAg

Selective detection of HBsAg (0–50 ng/mL) was conducted by spiking HBsAg in human serum, and the experiment was performed with modified ELISA. As shown in **Figure 4A**, the absorbance increased with HBsAg concentration, indicating the selective detection of HBsAg.

Specific experiment was conducted to confirm the reduction of false-positive results with four control experiments: (i) without dual probe; (ii) without secondary antibody; (iii) without HBsAg; and (iv) with control protein HCVcAg. To reduce the biofouling of biomolecules on the ELISA well, we added 2% BSA after attaching the probe to the surface. All other experimental

conditions were followed as described previously. As shown in **Figure 4B**, the absorbance level did not increase in the control experiments, but a clear increment of absorbance was recorded in the specific experiment. This result confirms that our modified ELISA specifically recognized HBsAg on the ELISA plate without any false-positive result.

4. Conclusion

HBV infection is a major health issue worldwide, causing various health problems, such as liver cancer. The early identification of this viral infection helps to suppress the spread of infection to other parts of the body. In this paper, we introduce a highly sensitive zeolite-modified ELISA plate to identify the HBV biomarker HBsAg. A dual probe of aptamer and antibody was attached to the surface of GNP and used as the capture probe, which attracted a greater number of HBsAg on the ELISA plate. Further, sandwich assay was conducted with monoclonal anti-HBsAg antibody, and a detection limit to 0.1 ng/mL was reached. Moreover, serum spiked experiments confirmed the increment of absorbance with an increase in HBsAg concentration, but the absorbance did not increase in the control experiments with complementary aptamer and control proteins, indicating the specific and selective detection of HBsAg. This nanomaterial-modified ELISA plate can detect low levels of HBsAg and help diagnose HBV infection in its early stages.

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Conflict of interest

Authors have no competing interests in relation to the publication of this article.

Author contributions

Conceptualization: Huijuan Geng, Wenyan Niu
Formal analysis: Huijuan Geng, Subash C.B. Gopinath
Investigation: Huijuan Geng and Wenyan Niu
Writing – original draft: Huijuan Geng and Wenyan Niu
Writing – review & editing: Huijuan Geng, Subash C.B. Gopinath, Wenyan Niu.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

Data will be provided on request to the corresponding author.

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