



Development of amperometric biosensor based on cloned hemagglutinin gene of H1N1 (swine flu) virus

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Abstract

The recent emergence of respiratory viruses especially COVID-19 and swine flu has underscored the need for robust and bedside detection methods. Swine flu virus is a very infectious virus of the respiratory system. Timely detection of this virus with high specificity and sensitivity is crucial for reducing morbidity as well as mortality. Cloning of gene segments into a non-infectious agent helps in the development of detection methods, vaccine development, and other studies. In this study, cloning was used to develop a biosensor for H1N1 pdm09 detection. A segment of the hemagglutinin gene was cloned in a vector and characterized with the help of colony touch PCR and blue–white screening. The recombinant plasmid was extracted, and the gene segment was confirmed with the help of HA-specific primers. A 5' amine group-attached hemagglutinin (HA) gene-specific DNA probe was immobilized on the working gold electrode surface to make a quick, specific, reliable, and sensitive detection method for H1N1pdm09 virus in human nasal swab samples. The HA probe was immobilized on the cysteine applied gold electrode of the screen-printed electrode through 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS). Differential pulse voltammetry was performed with the help of methylene blue, which is a redox indicator for the detection of single-stranded cloned HA gene segment. The developed sensor depicted high sensitivity for the H1N1 influenza virus with a detection limit of 0.6 ng ssDNA/6 μ l of the cloned HA sample. Specificity was also checked using H3N2 virus, *N. meningitides*, influenza A and positive H1N1pdm09 samples.

Keywords H1N1 · Hemagglutinin gene · Biosensor · *E. coli* DH5 α · TA cloning

Introduction

Every year H1N1 (swine flu) is the major cause of respiratory infections and deaths; amid the COVID-19 pandemic, its cases have been reported from different parts of the world as data provided by the World Health Organization (WHO, report). In India, 29,930 cases were reported along with 1236 deaths as per the latest National Centre for Disease Control (NCDC) data till February 2020 (NCDC 2019). The H1N1

virus (swine flu) is a subtype of influenza A, a member of the *Orthomyxoviridae* family with 4 types A, B, C, and D. Influenza types A and B cause infections, while C and D cause minor infections in animals. The H1N1 virus genome contains 8 negative-sense ssRNA components and encodes information of different proteins using host cell machines. There is 18 hemagglutinin (HA) types and 11 neuraminidase (NA) types of virus prevalent across the globe based on major surface antigens hemagglutinin (HA) and neuraminidase (NA) (Palese and Shaw 2007; CDC 2010). In the twenty-first century, first pandemic occurred in 2009 when a new H1N1 emerged as a pandemic strain which was evolved due to quadruple reassortment; also having a descendent of the 1918 H1N1 strain (Pandemic (H1N1) 2009-Weekly update 112, 2010, WHO Influenza update 365, 2020, Babakir-Mina et al. 2009; Garten et al. 2009). Symptoms similar to seasonal influenza are confusing, but some differentiating factors are fever above 104°F for more than 3 days, coughing, vomiting, dyspnea, severe dehydration, chest pain, headache, hypotension, nausea, and sore throat (PIB 2018, Lim

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and Mahmood 2011). Currently, detection can be done with molecular and serological diagnostic methods, i.e., reverse transcription loop-mediated isothermal amplification (RT-LAMP), rapid influenza detection test (RIDT), enzyme-linked immunosorbent assay (ELISA), conventional PCR, and RT-PCR (WHO 2017; Sharma et al. 2018; Al-zobaei 2012; Reber and Katz 2013; Pedersen, 2014). The severity of symptoms in patients augments with the due course of the disease, so timely detection is very important. Development and standardization of detection methods may involve the risk of infection while handling the virulent virus therefore, cloning the antigenic gene to generate protein for vaccine development or to develop a detection assay using the cloned gene is very helpful. That is why in this study partial gene fragment of a surface protein of the H1N1 virus was cloned in the *E. coli* DH5 α . The hemagglutinin gene (HA) is a surface protein of the H1N1 virus responsible for receptor binding on the epithelial cells of respiratory cells. The human influenza H1N1 pdm09 virus can interact with the α -2,6 glycosidic linkage of sialic acid with the help of HA protein. This cloned gene was used to develop a genosensor using a three-electrode system screen-printed gold electrode. Biosensors can detect a pathogen very quickly with high specificity. A number of sensors are present for various pathogens. But clone gene-based DNA sensor is a new strategy to develop a sensor on infectious agents. In 2012, a microfluidic methodology-based immunosensor was developed by Lee et al. (2012). A little while back, a DNA probe-based sensor was developed using a glassy carbon electrode changed with GCE (phenyl carboxylic acid) (Hyo et al. 2014). In 2019, a fluorophore-labeled antibody was used to bind with an antigen which results in a fluorescent signal after quenching the dye from the antibody (Jeong et al. 2018). Now CRISPER (Clustered regularly interspaced short palindromic repeat)-based methodology is providing an interesting technique to develop DNA sensors. CRISPER along with its associated multidomain protein Cas12a which is an endonuclease/nuclease/integrase acts as genetic scissors used for genetic engineering. CRISPER is a repeated palindromic DNA sequence in bacteria and archaea genome material that helps bacteria in maintaining antiviral activity. Foreign DNA is inserted with the CRISPER locus which acts as a recognition element for a complementary sequence in DNA sensors (Dronina et al. 2022). Cas9 protein extracted from *S. thermophilus* is a multidomain protein along with CRISPER used for gene manipulation or in gene therapy (Dronina et al. 2021). A pulsed amperometric-based sensor was developed for the detection of the bovine leukemia virus. Platinum wire was used as an electrode coated with polypyrrole entrapped single-stranded DNA. Pulsed amperometry was measured along with impedimetric analysis and PAD results were significant with EIS. It is a simple and sensitive method for DNA detection (Ramanaviciene and

Ramanavicius, 2004). Thus, all the available methods are time taking, costly, and also have chances of infection. There can also be a chance for highly pathogenic organisms to leak out from the laboratory and spread infection. In the present study, HA gene-based sensor is developed after cloning the gene in a vector. The sensor is based on the immobilization of the NH₂ group-linked probe on the working electrode with the help of cysteine. Then, complementary binding of probe (ssDNA) specific for HA gene of H1N1 pdm09 strain with the complementary cloned DNA of the H1N1 virus. The thiol group of cysteine can be absorbed on the gold surface of the electrode and the carboxyl group can bind with the probe. EDC-NHS was used to link between the -COOH group of cysteine and the -NH₂ group of the probe. When the cloned gene sample has a complementary DNA sequence representing a target for a pre-immobilized probe, a hybridization reaction takes place and results in dsDNA at the surface of the working electrode. A redox indicator methylene blue (MB) was used for the electrochemical study (Dash et al. 2012; Singh et al. 2017). This study explains a non-infectious way to develop a detection method with the help of non-infectious microbes. Through cloning expression of the gene and its further use for diagnostic can be achieved.

Materials and methods

Materials

Escherichia coli DH5 α , Thermo InsTA cloning kit, Purification kit, primers, LB broth, X-gal, ampicillin, IPTG, autoclave, petri dish. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), Methylene Blue, cysteine, *N*-hydroxysuccinimide (NHS), glucose, Tris-Cl, NaOH pellets, SDS, potassium acetate, glacial acetic acid, EtBr and agarose powder were bought from Sigma Aldrich, USA. Tris(hydroxymethyl) aminomethane (Tris base), sodium di-hydrogen ortho-phosphate, ethanol, EDTA (di-sodium hydrogen ortho-phosphate, ethylenediaminetetraacetic acid), and other materials were purchased from Qualigens. Viral RNA was isolated from samples at microbiology department, PGIMS, Rohtak. All samples were tested using Real-Time PCR (Rotor-Gene Q) from QIAGEN at PGIMS, Rohtak. Complementary DNA was synthesized using Thermo scientific (K1621) and HA gene probe 5'-NH₂-linked (GACACTGTAGACACA GTACTAG) was developed by Bio India Life Sciences, India. SPGE (Screen printed gold electrodes-AT220) were obtained from Dropsens. Primer 3 and BLAST were used for primer designing. UV illuminator (MAESTRO GEN) and Bio-Rad gel documentation were used for gel analysis.

Processing of samples and synthesis of cDNA

Nasal swab samples of patients were tested at microbiology department of PGIMS, Rohtak in BSL-2+ facility. QIAamp Viral RNA Mini kit was used for isolation of RNA at the microbiology department of PGI, Rohtak, and complementary DNA was prepared using random primers provided in the thermoscientific RevertAid First Strand cDNA Synthesis Kit (K1621). All the samples were also tested through conventional PCR using influenza A-specific matrix gene and the H1N1 subtype-specific hemagglutinin gene recommended by WHO (WHO, report). Selected primer set was specific only for H1N1pdm09 virus. All these steps and sensor development were performed at Maharshi Dayanand University, Rohtak. A set of primers specific to the HA gene were also designed using primer 3 software and used for testing the samples.

Random hexamer primers were selected for the synthesis of cDNA through the Agilent SureCycler PCR machine following these reaction mixtures (RNA-5 µl, Random primer-1 µl, nuclease-free water-6 µl, 5× reaction buffer-4 µl, Ribolock RNase inhibitor-1 µl, 10 mM dNTP mix-2 µl, Revert Aid M-MuLVRT-1 µl) following these temperatures (25 °C for 5 min, 42 °C for 1 h and terminated the reaction at 70 °C for 5 min). It is stored at -20 °C for further use as a sample in conventional PCR. Designed primers were used for amplification of the target region in HA gene segment 4 of the H1N1 virus using this cDNA.

Primer synthesis and characterization and standardization of PCR

HA gene was chosen because of its importance in virus entry into the human body. This surface antigen attaches with the sialic acid of epithelial cells of the respiratory system. HA gene sequences were taken from the influenza research database for 10 years and aligned using mega X software. The phylogeny tree was studied from the global influenza surveillance and response system. Gene is conserved and suitable for primer synthesis. NCBI primer tool is used for primer synthesis and a set of primers was selected for HA gene amplification. PCR is optimized for annealing temperature so that only the HA gene will be amplified.

Cloning of HA segment

After the amplification of the partial HA gene segment, the amplified segment was cloned in a Host *E. coli* DH5α. It was done with the help of the Ins TA cloning kit (Thermo Scientific). A linear segment was inserted into the vector pTZ57R/T. In polymerase chain reaction, Taq DNA polymerase adds a single 3'-A overhang to both ends of the amplified product, it can be easily cloned in a linear vector

having 3'-ddT overhang. T4 DNA ligase seals the nick present between the cloned segment and the vector. Young cultured host bacteria were grown in LB broth first and utilized in cloning after making it competent for cloning.

Screening of a cloned segment

After the process of cloning the partial HA gene segment, screening was done with the blue-white colony. In cloning, we have used *E. coli* DH5α which has a lacZΔM15 mutation. While screening an X-gal, which is a chromogenic substrate for enzyme β-galactosidase yields a blue color after digestion by bacteria into 5-Bromo-4-chloro-indoxyl. This product hybridizes spontaneously into a dimer 5,5'-dibromo-4,4'-dichloro-indigo which gives a blue color to a Petri dish. IPTG (Isopropyl β-D-1-thiogalactopyranoside) is an analog of galactose and acts as an inducer for the enzyme β-galactosidase. Recombinant bacteria cannot produce β-galactosidase and are unable to digest X-gal. That is why IPTG and X-gal both are used in the screening of clones. A recombinant host produces white colonies and a non-recombinant produces blue colonies which can be interpreted very easily.

Characterization of clone via Colony touch PCR

Colony touch PCR is a method to check the cloned segment direct from the culture with the help of PCR. A 4 µl of cultured sample broth was taken as a sample and heated to 95 °C for 5 min and used as a sample during PCR amplification. The same result is produced when we use cDNA for amplification. A band product of ~250 bp was produced as produced in conventional PCR.

Sequencing of the HA segment

The amplified clone HA gene segment was sequenced with the forward primer after the purification of the sample and aligned with other NCBI submitted HA gene segments. A 100% similarity was found in BLAST also. This alignment signifies the presence of a partial HA gene segment in the vector.

Plasmid isolation and sensor development

For plasmid isolation, white colonies were picked and cultured for 12–18 h in LB broth holding 50 µg/ml of ampicillin. 1.5 ml of culture was taken and spun down at 13,000 rpm for 14 min. The supernatant was discarded and 200 µl of solution I was added to maintain osmotically (50 mM glucose, 25 mM Tris-HCl pH 8.0, EDTA 10 Mm) and vortexed it smoothly. A volume of 400 µl of solution II (0.2 N NaOH, 1% SDS) was added to lyse the membrane

and shake gently, and put on the ice for 2 min. Then 300 μ l of chilled solution III (5 M potassium acetate, glacial acetic acid) was mixed, vortexed and left on ice for 5 min. At this step, the entire genomic DNA will remain denatured but the plasmid will renature, again centrifuged for 14 min at 13,000 rpm, taking the supernatant in a separate tube and discarding the remaining pellet. 0.6 ml of isopropanol was added and centrifuged for 14 min. The pellet was mixed in 300 μ l of 70% ethanol and discarded the supernatant. The pellet was dried and mixed with TE buffer to store it at $-20\text{ }^{\circ}\text{C}$. The extracted plasmid was seen in 1% agarose gel. The plasmid was used as a sample in PCR amplification also. A 243 bp size band was found which signifies the presence of an HA gene segment (Felicciello and Chinali 1993).

A biosensor is developed using a three-electrode system screen-printed electrode gold (SPGE) electrode. It has a working electrode and a counter electrode made up of gold and the reference electrode is made up of Ag/AgCl. The working electrode surface was chemically treated with 6 μ l of Cysteine (5 mM) for 12–24 h to develop a stable layer of cysteine. It has $-\text{SH}$ groups that can bind with the gold surface of the working electrode. The electrode surface was cleaned via autoclaved distilled H_2O to take out unbound Cysteine and then dehydrated at $25\text{ }^{\circ}\text{C}$. The equimolar solution of 10 mM EDC and NHS (1:1v/v) in autoclaved Milli-Q water was prepared and kept on the electrode for 1 h to activate the $-\text{COOH}$ group of Cysteine. Extra solutions washed away with autoclaved distilled H_2O . A 6 μ l of 5' amine group-linked 10 μM probe was applied for 1 h on the activated working electrode surface to make an amide bond between $-\text{COOH}$ groups of Cysteine and $-\text{NH}_2$ groups of probe via EDC/NHS solution. The modified electrode surface was washed 3–4 times with autoclaved distilled H_2O to remove the extra probe and dried at room temperature.

Results and discussion

H1N1 is a very contagious disease and every year infects millions of people worldwide. Cloning is a method to cultivate the antigenic segment for vaccine development and also for other virus studies. But cloning can also help in the development of a biosensor for the detection of the H1N1pdm09 virus. It is a safe method because after cloning, the chances of infection are negligible as the gene segment of interest is cloned in a non-infectious agent. Some studies are there based on the cloning of virus gene segments for different purposes. In 2020, cloning in the plasmid was performed to produce vaccine strains in Vero cells in large numbers (Liu et al. 2020). A chimeric HA gene-based vaccine is also designed to provide a universal vaccine against influenza viruses for long-lasting immunity based on cloning (Nachbagauer et al. 2021). In this study, cloning of HA gene

into *E. coli* DH5 α makes it non-infectious for the development of biosensors.

Primer synthesis and PCR characterization

All samples were checked with matrix gene and HA gene primers, the sequence was taken from WHO published documents to detect only H1N1pdm09. Matrix gene confirms the influenza A type specificity and the HA gene confirm the H1N1 subtype. An H1N1-specific primer set was designed for the HA gene which amplifies 243 bp. This amplified product of the HA gene was used for cloning. In Fig. 1A, a band of 244 bp long specific for matrix gene is shown confirming the presence of influenza A virus and a 173 bp amplified product is shown in (B) for many samples. Thus, both primer sets are confirming the presence of the H1N1pdm09 virus. HA gene-specific primer was synthesized and optimized for partial amplification of 243 bp of HA gene as shown in Fig. 1C.

Cloning of HA gene

PCR product of HA gene segment was used for cloning in *E. coli* DH5 α . The screening was done with the blue–white colony. In Fig. 2A, blue colonies are representing non-recombinant bacteria and white colonies are representing recombinant bacteria. Only non-recombinant bacteria can digest X-gal and yield a blue colony. Cultures are grown in LB broth containing ampicillin overnight and used for screening. Colony touch PCR was used for confirmation of the HA gene. Bacterial culture was heated at $95\text{ }^{\circ}\text{C}$ for 5 min to degrade the plasma membrane, so that genome and plasmid can come out. Then, this sample was used as a DNA

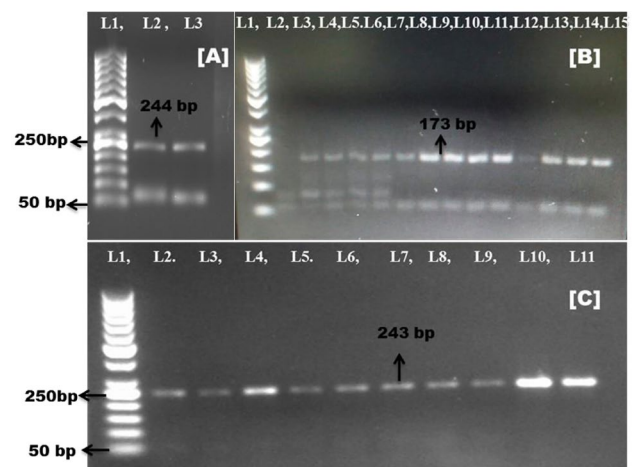
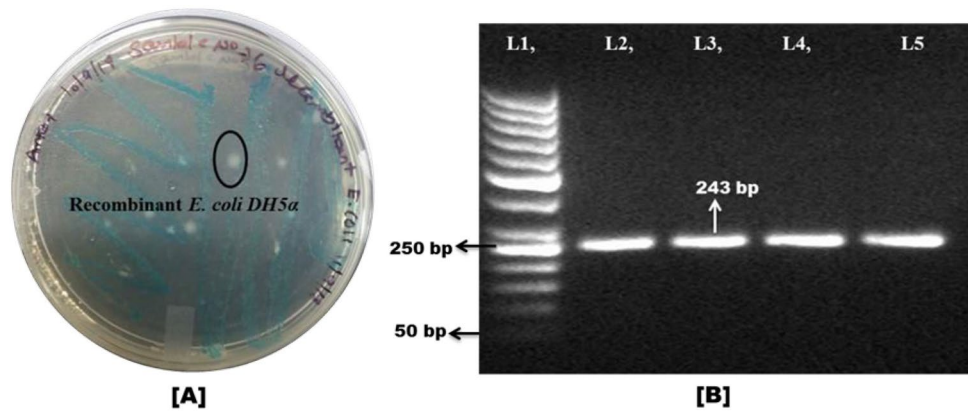


Fig. 1 A L1 is 50 bp ladder, lane 2, 3 – 244 bp matrix gene, B L1 is 50 bp ladder, L2 to L15 is showing 173 bp HA gene. C L1 is 50 bp ladder, L2 to L11 is showing 243 bp HA gene segment

Fig. 2 A Blue–white colony, white colony representing recombinant bacteria containing HA gene segment while blue colony representing non-recombinant bacteria. **B** L1–Ladder 50 bp, L2–L5 is showing result for 2% gel electrophoresis of colony touch PCR. A band of 243 bp of amplified product is shown in L2, L3, L4, L5



sample in PCR. The same conditions are used for amplification as in conventional PCR. A 243 bp band appeared in all wells.

Sequencing of cloned gene segment

An amplified product was used for sequencing. The forward primer was used for sequencing. The sequence was checked for its specificity with the help of BLAST and the sequence got similarity from 99.52 to 100% as shown in Fig. 3.

Plasmid isolation and gene characterization

To isolate the plasmid, a fresh LB broth was prepared and autoclaved. After that, inoculated with old broth and kept

at 37 °C overnight after adding 10 mg/ml ampicillin to the broth so that only recombinant bacteria will grow. The plasmid was isolated and used for sensor development. Plasmid extraction was confirmed using PCR. The extracted plasmid was used as a sample in HA gene-specific amplification. Nanodrop was also used for the quantification of the extracted product. A value of 260/280 was found 1.8 with different concentrations in different isolation batches in ng/μl. Gel electrophoresis was performed to check the amplified product as shown in Fig. 2.

Electrochemical analysis

An amine group-linked probe was attached to the electrode surface through EDC-NHS with cysteine. A probe

>Plasmid HA gene segment H1N1

AAGGAAGTACACACTCTGTTAATCTTCTGGAAGACAAGCATAACGGAAAACATGCAAACATAA
GAGGGGTAGCCCCATTGCATTTGGGTAATGTAACATTGCTGGCTGGATCCTGGGAAATCCAG
AGTGTGAATCACTCTCCACAGCAAGATCATGGTCTACATTGTGGAAACATCTAATTCAGACAAT
GGAACGTGTTACCCAGGAGATA → Reverse Primer



	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
✓	Influenza A virus (A/England/9779/2019(H1N1)) segment 4 hemagglutinin (HA) gene complete cds	Influenza A virus	377	377	97%	1e-100	99.52%	1701	MT639660.1
✓	Influenza A virus (A/England/9778/2019(H1N1)) segment 4 hemagglutinin (HA) gene complete cds	Influenza A virus	377	377	97%	1e-100	99.52%	1701	MT639659.1
✓	Influenza A virus (A/Ohio/9264/2019(H1N1)) segment 4 hemagglutinin (HA) gene complete cds	Influenza A virus	377	377	97%	1e-100	99.52%	1701	MT638916.1
✓	Influenza A virus (A/New York/9254/2019(H1N1)) segment 4 hemagglutinin (HA) gene complete cds	Influenza A virus	377	377	97%	1e-100	99.52%	1701	MT638868.1
✓	Influenza A virus (A/New Jersey/9240/2019(H1N1)) segment 4 hemagglutinin (HA) gene complete cds	Influenza A virus	377	377	97%	1e-100	99.52%	1701	MT638796.1
✓	Influenza A virus (A/Hawaii/9220/2019(H1N1)) segment 4 hemagglutinin (HA) gene complete cds	Influenza A virus	377	377	97%	1e-100	99.52%	1701	MT638716.1
✓	Influenza A virus (A/Arkansas/9170/2019(H1N1)) segment 4 hemagglutinin (HA) gene complete cds	Influenza A virus	377	377	97%	1e-100	99.52%	1701	MT638428.1
✓	Influenza A virus (A/Mississippi/10017/2019(H1N1)) segment 4 hemagglutinin (HA) gene complete cds	Influenza A virus	377	377	97%	1e-100	99.52%	1701	MT600075.1
✓	Influenza A virus (A/Georgia/10013/2019(H1N1)) segment 4 hemagglutinin (HA) gene complete cds	Influenza A virus	377	377	97%	1e-100	99.52%	1701	MT600051.1
✓	Influenza A virus (A/swine/Missouri/A02479312/2020(H1N1)) segment 4 hemagglutinin (HA) gene complete cds	Influenza A virus	377	377	95%	1e-100	100.00%	1701	MT554135.1
✓	Influenza A virus (A/swine/Iowa/A02479135/2020(H1N1)) segment 4 hemagglutinin (HA) gene complete cds	Influenza A virus	377	377	95%	1e-100	100.00%	1701	MT319783.1
✓	Influenza A virus (A/Michigan/484/2019(H1N1)) segment 4 hemagglutinin (HA) gene complete cds	Influenza A virus	377	377	97%	1e-100	99.52%	1752	MT303512.1

Fig. 3 HA clone gene sequence is shown after cloning the HA gene in the *E. coli* DH5α. The cloned gene is sequenced via Sanger sequencing and the result of the sequencing is showing 99.52% similarity in BLAST

consisting of oligonucleotide sequence which is highly specific to H1N1 swine flu (5'-NH₂-GACACTGTAGACACA GTACTAG-3') was used. Cysteine has-SH (thiol) group which will be adsorbed on the working electrode surface to make a stable sulfur-gold bond. This probe can make the covalent bond with cysteine after the formation of the layer of cysteine on the electrode surface using 1-ethyl-3-(3-dimethylamino propyl) carbodiimide/*N*-hydroxysuccinimide (EDC/NHS) chemistry (Kumar et al. 2012; Sheehan et al.

1961). The schematic presentation of the working scheme of fabricated working electrode Au/cys/ss-DNA (Probe) is shown in Scheme 1. The scheme is showing all the modifications that happen on the surface of the working electrode. Different dilution of sample DNA is made and used for the standardization of the sensor.

The sensor was also characterized by cyclic voltammetry at all steps, cysteine immobilization step, probe attachment, and cloned DNA binding step as shown in Fig. 4. Bare gold

Scheme 1 A schematic diagram of working scheme to develop a biosensor using recombinant *E. coli* DH5 α plasmid. A NH₂-linked probe is used for hybridization with ssDNA of recombinant plasmid for detection of H1N1 (swine flu)

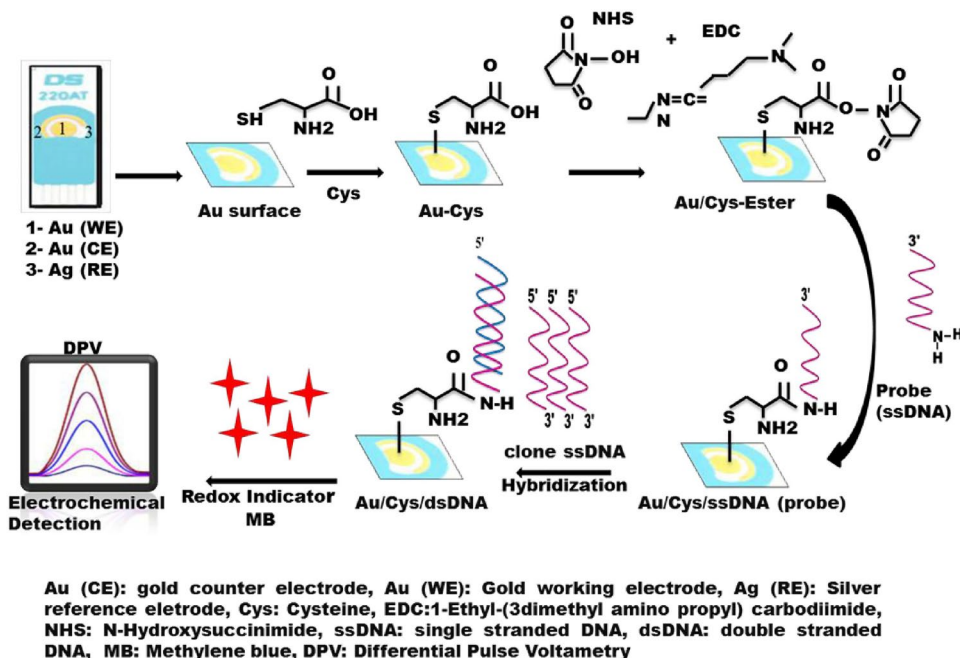
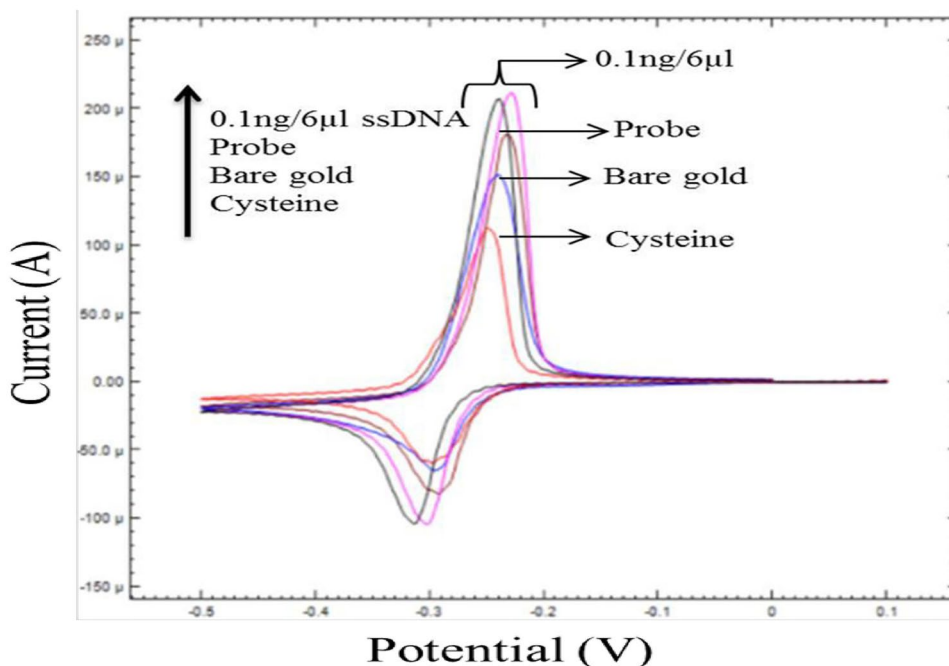


Fig. 4 Cyclic voltammetry of the surface of the screen-printed gold electrode for characterization at different steps of sensor development. Cysteine has lower value than bare gold. Gold is a good conductor of electricity. DNA is providing more sites for methylene blue binding and producing more due to redox reactions



has a higher current peak as compared to cysteine. Gold is a good conductor of electricity, producing more current with methylene blue. A complementary single-stranded DNA was applied for 10 min on the electrode surface. Extra DNA was removed by washing with TE buffer (10 mM Tris, 1 mM EDTA), pH 8.0, and PBS. An electrode was dried at room temperature before electrochemical analysis. A 50 μ l methylene blue was used as a redox indicator. After hybridization of the complementary sequence with the probe, the peak goes higher as shown in Fig. 4. Hybridized DNA is producing more current as compared to probe. Because double-stranded DNA has more nucleotide base pairs as compared to single-stranded DNA, methylene blue has more redox reaction sites to produce more current.

Plasmid DNA was extracted and the concentration of DNA was measured with nanodrop. A dilution range was prepared from 100 ng/6 μ l to 0.5 ng/6 μ l. A probe attached sensor was washed with autoclaved dH₂O to clean the extra unattached probe and dried at 25 °C for 5 min. All dilutions were heated at 95 °C for 5 min to make single-stranded DNA. A current difference was measured between all dilutions 0.5 ng to 100 ng/6 μ l of the sample via differential pulse voltammetry using electrochemistry (FRA2 μ Autolab type iii, Metrohm, India). Differential pulse voltammetry was measured at (\pm 25 °C) room temperature using a redox indicator 1 mM methylene blue (SIGMA-ALDRICH) prepared in 100 mM PBS, pH 7 in potentiostat (AUTO LAB). All prepared concentrations (0.5 ng/6 μ l to 100 ng/6 μ l) of the sample were incubated on the working electrode surface

for 10 min after washing with 1 mM HCl. An extra unbound sample was removed using TE buffer (10 mM Tris, 1 mM EDTA), pH 8.0 from the surface of the electrode.

The DPV image of all concentrations is shown in (Fig. 5A). The value of DPV peak current is lower for an unmodified gold electrode as compared to the probe applied surface. This may be possible because of the binding of methylene blue with the bases of ssDNA in the probe which increases the conductivity and also increase the value of current. The DPV current value for Au/cys/Probe/dsDNA was greater as compared to Au/cys/Probe/ssDNA and it increases with increasing the concentration of ssDNA sample until it achieves the stability at a higher concentration of sample as shown in Fig. 5. Methylene blue can bind with the extra unbound nitrogenous bases in DNA. Thus, an increase in the concentration of complementary ssDNA increases in DPV current value until all sites of the probe are occupied with a complementary sequence of DNA. The limit of detection (LOD) of the biosensor was calculated using the formula $LOD = 3(\sigma/S)$, where σ is representing standard deviation and S is representing calculated sensitivity. The L.O.D of the developed sensor is calculated as 0.6 ng/6 μ l ssG-DNA with (R^2) 0.98 regression coefficient (Fig. 5C). A hyperbolic curve was generated when a plot was drawn between different concentrations of ssDNA concerning their respective I_p values. The sensitivity (S) of this amperometric sensor is $\sim 10 \mu A cm^{-2} ng^{-1}$. It was calculated via the formula $S = m/A$, m representing the slope for the linear equation, and A is representing the surface area of the working electrode.

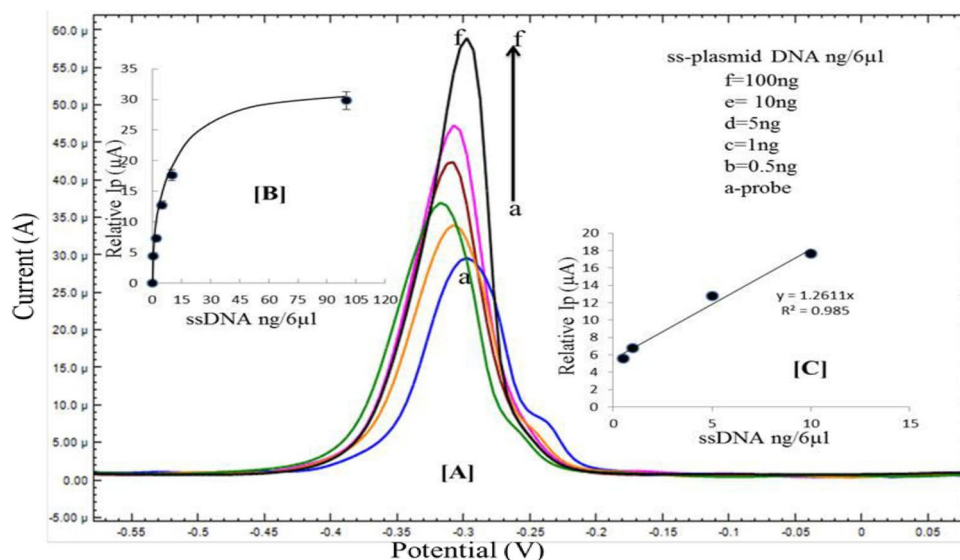


Fig. 5 DPV of **A** Au/Cys/Probe-ssDNA and (b–f) hybridization with 0.5 ng, 1, 5, 10 and 100 ng/6 μ l of recombinant plasmid having HA gene of H1N1 (Swine flu) ssDNA using redox indicator 1 mM methylene blue in 100 mM PBS, pH 7. The inset **B** shows hyperbolic curve between relative I_p (with respect to probe as zero) and increas-

ing concentrations of hybridizing ssDNA of cloned HA gene of H1N1 (Swine flu). The inset **C** shows 0.1–10 ng/6 μ l ssDNA region of the linear standard graph for calculation of limit of detection and concentration of unknown DNA sample for confirmation of the disease

The specificity of the developed amperometric sensor was checked using pathogens (*N. meningitidis*, H3N2, influenza A virus). A 6 μl of each sample 10 ng/6 μl was placed on the working electrode. The current was measured for all samples, the negative sample's current value was the same as the probe except H1N1 positive and recombinant plasmid clone for the HA gene. A rise in current value occurs only in H1N1 positive virus (10 ng/6 μl) and HA gene-positive recombinant plasmid as shown in Fig. 6.

This methodology is unique for fabricating a sensor without taking the risk of infection. Cloning of genes in vectors provides a safer way to do research in diagnostic studies. The developed sensor is very sensitive and specific for H1N1 pdm09. The screening of cloned gene was done at every step of cloning to avoid any unwanted insertion. Therefore cloned gene-based amperometric sensor was developed. Differential pulse voltammetry was selected for the amperometric study. In all popular pulse techniques, which include DPV, are more sensitive than the linear sweep strategies due to their minimized capacitive current. This minimized capacitive current helps in improving the sensitivity of the technique. Also, DPV is used in the case of slow kinetics reactions and irreversible reactions but other voltammetry like square-wave voltammetry is used in the case of reversible and fast reaction kinetics reactions. Cyclic voltammetry and linear sweep voltammetry, in both cases, potential changed with respect to time. In cyclic voltammetry, potential is scanned from a fixed potential at both electrodes in a cyclic way and returns to the initial value. The redox reaction occurs at an anodic and/or cathodic peak which might be proportional to the concentration of reacting species. Cyclic voltammetry is also sensitive but used mostly for

exploratory work. It is used in getting important information about the type of redox reactions, and reversibility of reactions but pulse voltammetry is used for quantitative analysis of reactions (Simoes and Xavier 2017; Patel 2021). Therefore, DPV was selected for the study to achieve better sensitivity in an irreversible reaction. Recently, an immunosensor was reported with the help of fluorophore-labeled anti-HA Fab which gives fluorescence after quenching of fluorophore as a response to binding of antigen with labeled antibody (Jeong et al. 2018). In 2022, another method was developed based on polyaniline and polypyrrole vesicles. An HA protein-specific peptide was attached to the surface of these vesicles which can bind with the H1N1 virus specifically. An optical response that is due to the presence of vesicles was recorded with the help of a microplate multimode reader (Park et al. 2022). A comparative analysis of some latest biosensors is presented in Table 1 in which most are based on antigen and antibody binding. A DNA-based sensor is more sensitive due to its complementary binding but the antigen–antibody-based method can give false-positive and -negative results.

Conclusion

The H1N1 virus is a big public concern for health and is responsible for millions of death throughout history. The virus takes 2–3 days for symptomatic appearance in the host body which makes the infected person's condition more severe. Therefore, early detection is important for timely treatment. Older methods are time taking, especially those based on the antigen–antibody titer, which makes them less

Fig. 6 Specificity of screen-printed gold electrode biosensor with rec-plasmid HA gene (10 ng/6 μl), H1N1 pdm09 (10 ng/6 μl), influenza A positive (negative sample 2) (10 ng/6 μl), *N. meningitidis* (10 ng/6 μl) and H3N2 virus 10 ng/6 μl of ssDNA is performed using redox indicator 1 mM methylene blue in 100 mM PBS, pH 7

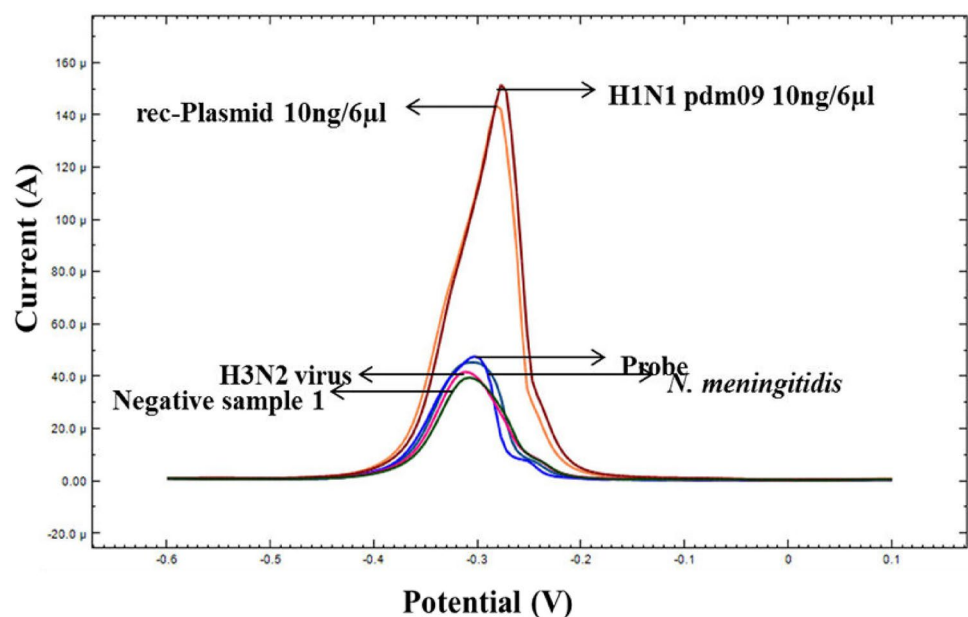


Table 1 A comparative analysis of biosensors for the detection of H1N1 pdm09 virus

Sr. no.	Sensor type	Gene/protein	Sensitivity/L.O.D	Sample type	Detection time	References
1	Fluorescent immunosensor	HA/fusion antibody	Not reported	H1N1 virus	–	Lee et al. (2012)
2	Immunosensor/SPR	HA/antibody	30 PFU/ml	Labeled anti-HA	20 min	Su et al. (2012)
3	FET biosensor	HA binding	6000HA mol/20 μ l	HA protein	–	Hideshima et al. (2013)
4	Immunosensor	M1/polyclonal antibody	80–100 virions/ μ l	H1N1 virus	30 min	Nidzworski et al. (2014)
5	PEDOT with galactose	HA binding	0.12, 0.013 HAU	H1N1 virus	–	Hai et al. (2017)
6	AuNP immunosensor	HA, NA antibody	50.5 pg/ml	H1N1 virus	–	Ahmed et al. (2017)
7	SiO ₂ -IO	HA antibody	10 ³ –10 ⁵ PFU	H1N1 virus	–	Lee et al. (2018)
8	DPM-coated gold electrode	His ₆ -H1 HA	1 \times 10 ⁹ to 1 \times 10 ⁸ fold	HA antibody in sera	–	Mikuła et al. (2018)
9	HA gene-based genosensor	HA gene	0.6 ng/6 μ l	Recombinant HA gene	30 min	Present study

HA hemagglutinin, NA neuraminidase, SPR surface plasmon resonance, PEDOT poly(3,4-ethylenedioxythiophene), AuNP gold nanoparticles, SiO₂-IO silicon dioxide, DPM dipyrromethene, HAU hemagglutinin unit, M1 matrix 1

sensitive and also gives false-negative results. Real-time PCR is the standard method for virus detection, but the equipment and other reagents are very expensive and cannot be afforded in every area of the world. Other methods for virus detection are rapid detection kits, virus culture, ELISA and biosensors; however, chances of spillover may expose the handler to infectious agent along with false-positive results. In the present study, the cloned gene-based amperometric sensor was developed using differential pulse voltammetry. It is a safer and easy way to detect the virus in 30 min. It is specific and sensitive to the H1N1pdm09 virus. This sensor can detect a L.O.D of 0.6 ng/6 μ l ssG-DNA with a sensitivity of $\sim 10 \mu\text{A cm}^{-2} \text{ng}^{-1}$. The method can be applied to other types of pathogens also.

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Declarations

Conflict of interest Authors declare no conflict of interest.

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