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Hemagglutinin gene based biosensor for early detection of swine flu (H1N1) infection in human

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ABSTRACT

Hemagglutinin (HA) is a glycoprotein found on the surface of influenza A subtype virus H1N1 which play a major role in infection to the human by binding the virus to cells with sialic acid on the membrane of upper respiratory tract or erythrocytes. Based on sequence of HA gene an impedimetric biosensor was developed by immobilizing amino labeled single stranded DNA probe onto cysteine modified gold surface of the screen printed electrode for early and rapid detection of H1N1 (Swine flu) in human. The electrochemical impedance was recorded after hybridization of probe with single stranded cDNA (ss-cDNA) of H1N1 patient samples in presence of redox couple. All available methods for detection of H1N1 including RT-PCR are either expensive or time consuming. However, impedimetric biosensor is not only highly specific for H1N1 virus but also can detect as low as 0.004 ng (limit of detection) ss-cDNA in 6 μL only in 30 min. The sensitivity of the sensor was 3750 Ω cm^{-2} ng^{-1} of DNA. The biosensor was well characterized using surface cyclic voltammetry, validated with patient samples and compared with existing methods. The sensor can be used in hospitals, diagnostic centres as well as in remote areas for early and rapid diagnosis.

1. Introduction

H1N1 (Swine flu) is a viral infection caused by influenza A subtype virus of *Orthomyxoviridae* family. It is responsible for major outbreaks from historical periods and also responsible for 2009 (H1N1) pandemic. It has single stranded negative sense segmented RNA genome which codes for 16 different proteins [1,2]. The shape of virus may be spherical or filamentous having size of 80–120 nm. Nucleocapsid of virus has symmetric helical structure and its surface has glycoprotein hemagglutinin (HA) and enzyme neuraminidase (NA). HA helps in attachment of virus through sialic acid of glycoprotein on the surface of upper respiratory tract or erythrocytes of host and enzyme NA cuts sialic acid from cell surface and free descendants of virus from infected patient cells [3]. On the basis of antigenic analysis, surface antigens HA and NA is classified into 18 types and 11 types, respectively [4]. These different segments makes new combination and responsible for outbreaks of influenza in population which lacking immunity. Pigs have receptors for both human influenza virus as well as avian influenza viruses and act as a site for genetic re-assortment of different segments of genes and responsible for antigenic shift. Thus pig acts as natural mixing vessel and responsible for interspecies transmission [5,6]. Infection in humans from avian influenza is restricted due to the unavailability of receptors that can bind to avian influenza viruses [7]. Infections due to the influenza A virus was reported in ancient history also but reliable data about H1N1 strain was reported during 1918 pandemic and after 1918 it was also confirmed that H1N1 can infect pigs and humans both when same H1N1 strain was detected in pigs as classical swine H1N1 in United States in 1930 [8]. A novel influenza virus was emerged in 2009 causing >18,000 deaths worldwide till August 2010. H1N1 2009 pandemic was a result of quadruple reassortment in triply assorted virus with Eurasian (Europe and Asia) swine virus. One of the viruses was descendent of 1918 strain [9–11]. Same strain was active continuously when it again caused 2015 pandemic worldwide, infected a large number of people in India causing many deaths [12]. A large number of infections and deaths also occur in 2016–17. The symptoms of influenza are mostly similar to that of seasonal influenza virus. It includes fever above 104 °F for more than three days, headache, coughing, sore throat, vomiting, chest pain, hypotension, severe dehydration and nausea [13]. Every year H1N1 causes death of thousands of people worldwide. There are many diagnosis methods such as viral culture, hemagglutinin inhibition test (HI), immunodiffusion test, rapid influenza diagnostic tests (RIDT), complement inhibition test (CI), enzyme linked immunosorbent assay (ELISA) and real time PCR (RT-PCR). But all these methods are time taking, less sensitive, non-specific on single

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test, expensive and cannot diagnose rapidly during an outbreak of the disease. Antigen based detection methods are not very sensitive. RT-PCR is very sensitive but LOD (limit of detection) of test is high and cannot detect less number of viruses. As severity of infection increases with time so, there is a need for early detection of H1N1 infection [14–22]. Biosensors are the modern diagnostic techniques having high sensitivity and specificity for pathogens. A plastic based microfluidic immunosensor was reported in 2012 by [23]. Recently, a DNA biosensor was also developed using phenyl carboxylic acid-modified glassy carbon electrode (GCE) by immobilization of DNA probe and hybridization with single stranded complementary DNA (ss-cDNA) of H1N1by measuring change in current [24]. Silanized surface based SiO_2-IO nanosensor was developed which provides good surface for attachment of anti-HA based antibody through biological linkers for detection of virus by checking shift in reflectance spectrum [25]. The above sensor was either less sensitive or time consuming.

A need of rapid, sensitive, specific and cost effective biosensors are presently required for the detection of H1N1 (Swine flu) in human. Electrochemical impedance (EI) spectroscopy based on DNA hybridization emerged a powerful diagnostic tool for diagnosis of diseases [26]. Recently, H1N1 virus specific impedimetric aptamer was also developed for multivalent binding with inactivated H1N1viruses [27]. But all these methods are very complex, expensive and cumbersome.

Therefore, present study focused on the development of electrochemical impedimetric biosensor by immobilizing HA gene specific ssDNA probe onto cysteine modified screen printed gold electrode and its hybridization with ss-cDNA of patient samples using redox couple potassium ferricyanide and potassium ferrocyanide $[Fe(CN)_6]^{3-/4-}$.

2. Experimental

2.1. Materials

Cysteine, EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide), NHS (N-hydroxysuccinimide), methylene blue and agarose powder were obtained from Sigma-Aldrich, USA. Trizma base (Tris (hydroxymethyl) aminomethane), EDTA (ethylenediaminetetraacetic acid), ethanol (100%), potassium ferricyanide and ferrocyanide, sodium dihydrogen ortho-phosphate, di-sodium hydrogen ortho-phosphate were purchased from Qualigens, India. All other chemicals were procured from local suppliers. QIAamp viral RNA mini kit (52906) was purchased from Qiagen, India. All buffers and Milli-Q water were autoclaved to prevent contamination due to foreign DNA. RT-PCR kit (4441240C) and cDNA synthesis kit (K1621) and 50 bp DNA marker (SM0371) were procured from Thermo Fisher Scientific, USA. 5′-NH_2 labeled HA gene probe (5′-NH_2-GACACTGTAGACACAGTA CTA G-3′) was acquired from Bio India Life Sciences. Forward and reverse primers were synthesized from Sigma-Aldrich. The screen printed gold electrode (SPGE) was obtained from Metrohm-DropSens, Spain and modified in the lab.

2.2. H1N1 samples collection and preparation of cDNA from RNA

The patient nasal swab and oropharyngeal samples were collected in virus transport media and processed in BSL-II facility for RNA isolation at PGIMS, Rohtak. The nasal 200 μl swab sample was mixed 1:1 (v/v) with lysis buffer (provided in kit) and RNA was extracted using QIAamp viral RNA Mini kit in QIAGEN QIAcube automated machine (SN 48159). The samples were further analysed using Real-Time PCR kit (RT-PCR) for confirmation of H1N1 (2009 pandemic) strain at PGIMS, Rohtak. The positive and negative (H1N1) samples were further used at Centre for Medical Biotechnology, M.D. University, Rohtak. The complementary DNA (cDNA) was made using random primers of cDNA synthesis kit (Thermo Scientific RevertAid First Strand). The confirmation of positive and negative samples of H1N1 were carried out using specific primers of 2009 pandemic matrix gene (MA) and HA gene (specific for H1N1). Matrix gene primers and HA gene primers were selected from revised WHO (World Health Organization) guidelines which was published in August 2009. Since, MA gene sequence is conserved for all types of influenza viruses therefore one set of primer specific for influenza A type was selected to confirm the presence of influenza viruses. Besides, HA gene primers specific for H1N1 (Forward primer 5′-GACACTGTAGACACAG TACTAG-3′, Reverse primer 5′-ATCTCCTGGGTAACACGTTC-3′) were selected for HA gene amplification using initial denaturation (95 °C for 8 min) followed 30 cycles of denaturation (95 °C for 30 s), annealing (55 °C for 30s), extension (72 °C for 1 min) and final extension (72 °C for 8 min) PCR. The amplified PCR product corresponds 250 bp marker in 1.5% agarose gel electrophoresis (Fig. 1[A],[B]) which was partially sequenced at IGIB using reverse primer and found 243 bp (Fig. 1[C]). The partial amplified sequence 243 bp was further BLAST (Basic Local Alignment Search Tool) and found approximately 99% homology with HA gene of H1N1.

2.3. Fabrication of the sensor

A screen printed gold electrode (SPGE) that contains gold (Au) as working and counter electrode and silver (Ag) as reference electrode was chemically modified for the development of the HA gene specific impedimetric biosensor. The working circular (diameter, 4 mm) Au electrode surface area was calculated using formula πr^2 ($\pi = 3.14$ and radius (r) = 0.2 cm) and found 0.126 cm^2. The cysteine (5 mM in Milli-Q water) 6 μL was placed on the working area of Au electrode (0.126cm^2) for 10-18 h to create fixed monolayer of cysteine. The cysteine can self-assemble with Au electrode surface through thiol (-SH) groups. Then, the electrode was carefully washed with Milli-Q water to eliminate excess unbound cysteine and dried at room temperature (25 °C).The 5′-amine labeled 22-mer ssDNA probe (5′-NH_2-GACACT GTAGACACAGTACT AG-3′) of HA gene (20 μM in Milli-Q water) was diluted (v/v) 1:1 with 10 mM EDC and 10 mM NHS (in Milli-Q water) to make10 μM the final concentration of the probe. Then, 6 μL of the above mixture (probe/EDC-NHS) was put onto the working area of Au electrode (0.126 cm^2) for 2 h so that amide (CO-NH) bond could form between the carboxyl group of the cysteine and amino group of the probe (EDC-NHS chemistry) [28,29]. The electrode was washed 4–5 times with Milli-Q water to eliminate extra unbound probe and then dried to take electrochemical impedance and cyclic voltammetric (CV) measurements for surface modification study (Fig. S2, Supplementary material).

2.4. Hybridization with ss-cDNA

The HA gene specific NH_2-linked ssDNA probe attached on working Au electrode surface as described earlier. The cDNA of H1N1 was heated at 95 °C for 5 min to make ss-cDNA for hybridization with complementary ssDNA probe. The different concentrations of ss-cDNA (0.1 ng–400 ng/6 μL) in Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 8.0) were used for hybridization with probe for 10 min. The hybridization time was standardized as shown in Fig. S1 (supplementary material). After hybridization, the electrode was continuously washed several times with TE buffer, pH 8 (to eliminate unhybridized DNA) succeeded by PBS (50 mM sodium phosphate buffer with 0.9% sodium chloride) of pH 7.0 and dried before electrochemical impedance and CV measurements for confirmation of modified electrode surface using methylene blue (MB) as redox indicator (Fig. S2, Supplementary material). The schematic fabrication of Au/Cys/ssDNA (Probe) and hybridization with ss-cDNA of H1N1 to form Au/Cys/Probe/dsDNA is shown in Scheme 1.

2.5. Electrochemical impedance measurements

The electrochemical impedimetric (EI) study were performed using 50 μL of 5 mM redox couple potassium ferricyanide and ferrocyanide [$Fe(CN)_6)^{3-/4-}$] in PBS, pH 7.0 at screen printed electrode attached with

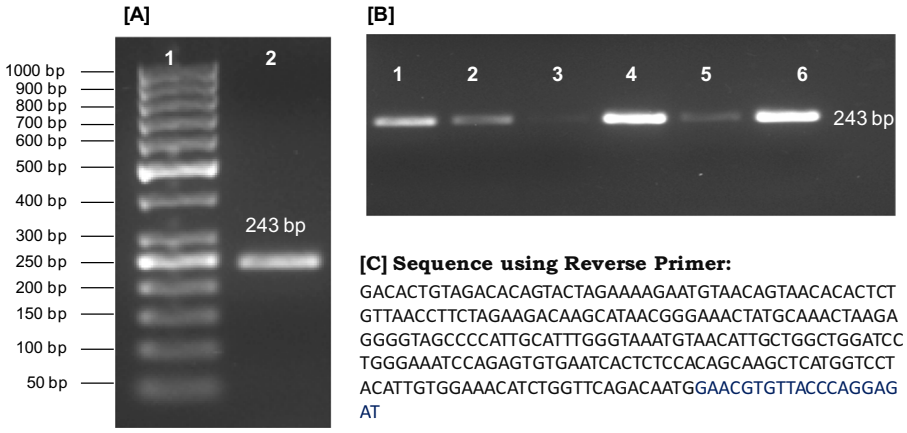


Fig. 1. [A] 1.5% Agarose gel electrophoresis of PCR product under UV illumination. Lane-1: 50 bp DNA ladder (marker), lane-2: 243 bp HA gene amplicon of PCR product. [B] Amplified PCR product of 243 bp (HA gene of H1N1) for different positive patient samples in lane 1–6 in 1.5% agarose gel using self-designed primers. [C] Partial sequence of PCR product using reverse primer of HA gene.

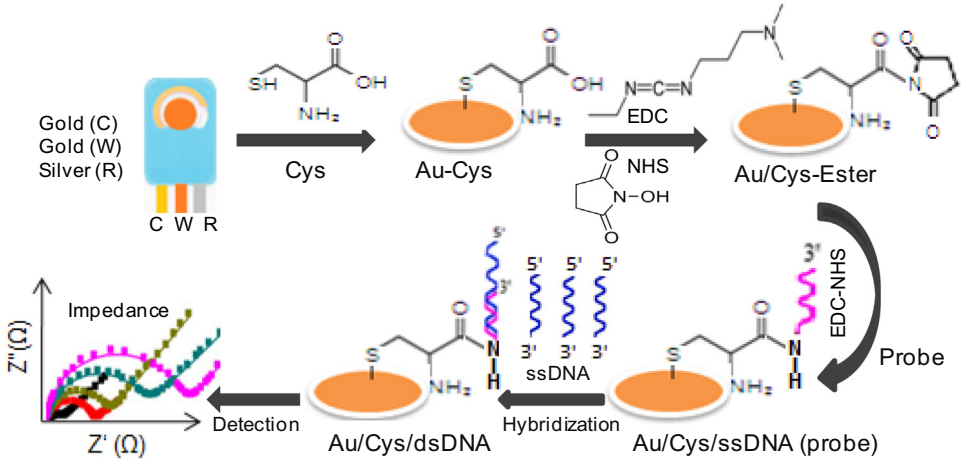
potentiostat/galvantostat (FRA2 μAutolab type iii, Metrohm, India) at room temperature. The EI of Au/Cys/Probe was measured as control and similarly after 10 min hybridization with different concentrations of ss-cDNA (0.1–400 ng/6 μL) with ssDNA probe in TE buffer, pH 8 at working Au electrode surface.

After hybridization, Au/Cys/dsDNA electrode was washed 3–4 times with TE buffer, pH 8.0, followed by similarly with PBS, pH 7.0 and dried before taking electrochemical impedance at frequency range 10^{-1}–10^{-5}Hz.

3. Results and discussion

3.1. Impedimetric studies

The impedance studies of Au/Cys/ssDNA(Probe) and Au/Cys/Probe/dsDNA of Nyquist plot for is shown in Fig. 2[A]. The R_{ct} (resistance charge transfer) is mostly measured as diameter of the semicircle interface of the Au electrode [30,31]. Nyquist complex impedance (EI) study of electrode fit to Randles (equivalent) circuit of electrochemical interface where, R_s: electrolyte resistance, C_{dl}: double layer capacitance, R_{ct}: charge transfer resistance and W_d represents the Warburg diffusion element (Fig. 2, inset). C_{dl} is found in series to R_s and parallel with R_{ct} [26]. The R_{ct} of unmodified Au surface working electrode was measured lower in comparison of Au/Cys/ssDNA(Probe) (data not shown). This might be due to increased conductivity (increase in current) of the Au which causes decrease in the impedance. The R_{ct} of Au/Cys/dsDNA was found greater than Au/Cys/ssDNA (probe) and increases with increasing ss-cDNA of hybridizing concentrations and finally saturated at higher ss-cDNA concentrations of H1N1. This is observed due to the presence of more phosphate groups (negatively charged) on the working area of Au electrode after hybridization which subsequently prevented the $[Fe(CN)_6]^{3-/4-}$ ions from reaching the electrode surface which causes an increase in the R_{ct} value and finally saturated (no increase in R_{ct} value). The relative R_{ct} values of different concentrations of ss-cDNA with respect to probe (control) as zero is plotted as shown in Fig. 2[B]. This confirms that at lower concentrations of hybridizing ss-CDNA is directly related to signal (first order rate of reaction) before saturation of probe ssDNA but after saturation no change in signals



Cys: Cysteine, Au: Gold, EDC:1-Ethyl-3-(3-dimethyl aminopropyl) carbodiimide, NHS: N-Hydroxysuccinimide, ssDNA: single stranded DNA, dsDNA: double stranded DNA, C: Counter, W: Working, R: Reference electrode

Scheme 1. Schematic fabrication of gene specific impedimetric biosensor for detection of swine flu (H1N1) in human.

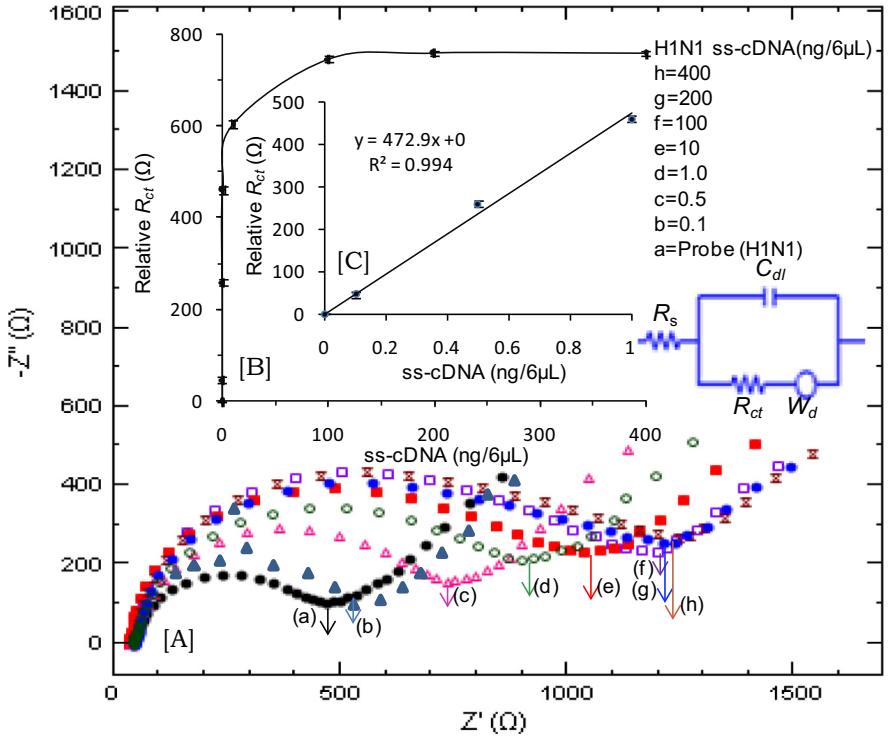


Fig. 2. [A] EI spectra at frequency range of 10^{-1}–10^{5} Hz and 0.01 V amplitude of (a) Au/Cys/ssDNA (Probe) and (b–g) after hybridization with 0.1, 0.5, 1.0, 10, 100, 200 and 400 ng/6 µL of H1N1 (Swine flu) ss-cDNA using redox couple 5 mM potassium ferricyanide and ferrocyanide ($[Fe(CN)_6]^{3-/4-}$) in 50 mM PBS, pH 7. The inset [B] shows hyperbolic curve between relative R_{ct} (with respect to probe as zero) with increasing concentrations of hybridizing ss-cDNA of H1N1 (Swine flu). The inset [C] shows 0–1 ng ss-cDNA/6 µL region of the linear standard graph for calculation of limit of detection (LOD).

[30,32].Therefore, we have used initial lower concentrations at which signal changes with increasing concentrations [regression coefficient $R^2 = 0.994$) for calculation of LOD and sensitivity (Fig. 2[C]). The sensitivity (S) and LOD of the biosensor experimentally was found 3750 $\Omega\ cm^{-2}\ ng^{-1}$ of ss-cDNA and 0.004 ng ss-cDNA, respectively from standard curve (Fig. 2[C]) using general formula S = m/A, where 'm' representing slope of linear equation of line and 'A' is working surface area of Au (0.126 cm^2). LOD of the biosensor was determined using method 3(σ/S), where 'σ' is representing the standard deviation and 'S' is for the sensitivity.

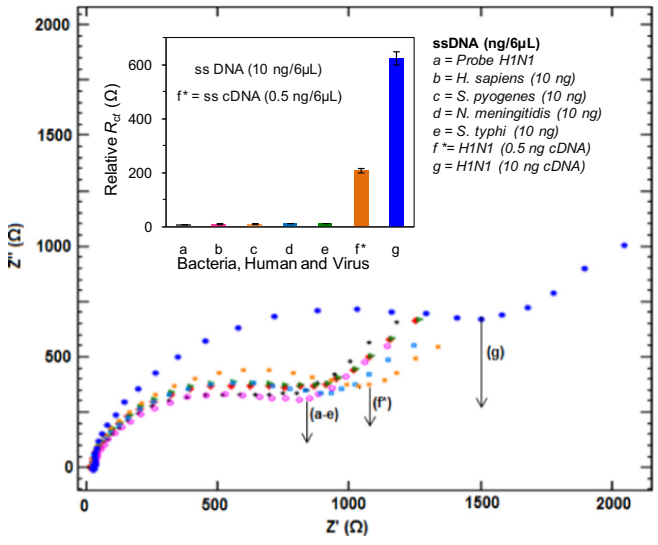


Fig. 3. Specificity of the impedimetric biosensor with H1N1, human DNA and other possible pathogens causing infections in human. The inset shows the relative R_{ct} value (with respect to the immobilized probe as zero) after hybridization with ss-cDNA with other possible pathogens and human (10 ng/6 µL) and H1N1 (0.5 and 10 ng/6 µL).

3.2. Specificity studies

The specificity of the gene specific impedimetric sensor with H1N1, human DNA and other possible pathogens (*S. typhi*, *N. meningitidis*, *S. pyogenes*) is presented in Fig. 3. The R_{ct} values of the sensor after hybridization of probe with 10 ng/6 µL of ssDNA of other infectious pathogens (bacteria) were almost similar as the control (probe) R_{ct} except with H1N1 which showed higher R_{ct} even at low concentration (0.5 ng/6 µL) after hybridization with ss-cDNA (Fig. 3 inset). The prominent increase in R_{ct} value was acquired only in the case of H1N1, which confirms the specificity of the impedimetric biosensor only to H1N1 virus. This may be due to strong specificity of the probe only to the HA gene of H1N1, no other pathogens including human DNA.

3.3. Surface CV studies

The working Au electrode surface was characterized at different stage of modifications before and after hybridization with ss-cDNA of H1N1 positive samples using cyclic voltammetry (CV) (supplementary material, Fig. S2).

3.4. Validation of the sensor

The fabricated biosensor was validated with 13 suspected H1N1 patient samples after preparing cDNA from RNA (as described above) from PGIMS, Rohtak hospital using electrochemical impedance study (EIS) with respect to HA gene specific probe (Fig. 4). The cDNA of suspected patient of H1N1 samples were heated at 95 °C for 5 min to denature (ds-cDNA) and hybridized as described earlier with immobilized HA probe for 10 min. The R_{ct} value of sensor differ in accordance with HA probe, negative control (normal nasal swab without H1N1) and positive

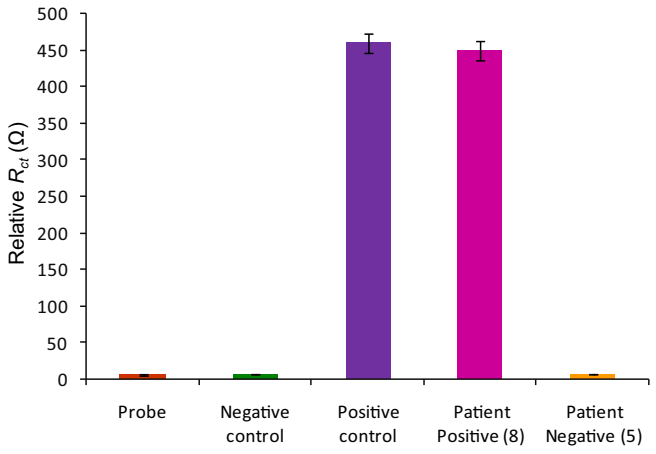


Fig. 4. Relative R_{ct} value of EIS after hybridization with ss-cDNA from suspected H1N1 patient samples. Nasal swab of 13 suspected patient samples were obtained and cDNA was made from RNA of H1N1 virus as described earlier. The samples (ss-cDNA) were hybridized with ssDNA probe and relative R_{ct} values were obtained for validation of the sensor.

control (swab with H1N1, 1.0 ng/6 μL), which acts as threshold for H1N1 patient samples. In (Fig. 4) y-axis represents relative R_{ct} in EIS with respect to probe (control), negative control without DNA in sample to hybridize with probe (no change in R_{ct}), positive control contains ss-cDNA of H1N1 (1.0 ng/6 μL) which was hybridized with HA probe and increased in the R_{ct} with respect to probe. The suspected H1N1 patients ss-cDNA were quantified by nanodrop spectrophotometer (1.0–1.2 ng/6 μL), hybridized with HA probe and relative R_{ct} was measured impedimetrically. The average 8 positive and 5 negative samples R_{ct} values are plotted with respect to above described controls. Negative patient do not have H1N1 (no complementary ss-cDNA/RNA) but may contain other pathogens (non-complementary DNA or even no RNA) therefore, no significant increase in R_{ct} value. Negative sample of patients are negative due to their R_{ct} value correspond to negative control whereas, positive patient samples (with approximately same ss-cDNA hybridizing concentrations as used in positive control) R_{ct} value correspond to positive control (±10–12% variation in average R_{ct}). The usual methods of H1N1 detection through RT-PCR (4–5 h) and 1.5% agarose gel electrophoresis with markers (3–4 h) were also performed in the lab (Fig. 5) for confirmation of the swine flu disease and the results were found comparable with the present impedimetric biosensor. The designed probe of our biosensor is derived from HA gene sequence of H1N1 which also depicting 100% homology only with H1N1 and do not shows any similarity with other pathogens or human on BLAST (Basic Local Alignment Search Tools) confirms the specificity of the impedimetric biosensor only with H1N1 virus. H1N1 is an infectious disease responsible for many deaths worldwide every year so early and rapid detection is a primary concern to save life of several infected people during an outbreak of the disease. There are many diagnostic methods are reported for H1N1 detection (Table 1) but these are either less sensitive, expensive, time consuming or non-confirmatory on single test. The present biosensor is specific only for H1N1 and probe can be modified if any mutations are found in genes of H1N1without changing fabrication techniques.

3.5. Stability and re-usability

The immobilized H1N1 probe on modified screen printed electrode was kept at 4 °C for 4 months which showed only 5% loss in initial relative R_{ct} value (data not shown). Further, stability is under monitor. The biosensor can't be re-used as patient samples are from infectious virus. Therefore, screen printed Au electrodes are used and destroyed after single use.

3.6. Advantage of the present sensor

The comparison of all available methods for detection of H1N1 (swine flu) is summarized in Table 1. The culturing of virus is cumbersome, time consuming (7 days) and detection based on direct immunofluorescence tests with specific antibody against influenza A virus nucleoprotein on fixed cell smears [33]. Real time PCR takes >4 h and requires multiple fluorescent labeled DNA probes for detection. Most of the labs used this method according to WHO guidelines [33]. Complement fixation test is based on antigen-antibody reactions and use 8 units of HA antigen for all different concentration of antisera. The disadvantage is that it gives false negative antibody responses, takes 24 h and poor sensitivity and specificity [20]. In double immunodiffusion test >32 HA titre cultured virus used in allantioc fluid (infected embryonated chicken eggs) and takes detection in 4–5 days. It requires 10^7 HA units/mL reference antigen and 150 μg/ml reference antisera [14]. ELISA is a good alternative tool when number of cases is large during an outbreak and RT-PCR not available. It is based on throat swab viral load and cut-off value of viral load was between 10^5 and 10^6. The higher the viral load, higher will be the sensitivity. The sensitivity was found lower at early stage of infection due to lower viral load [34]. Direct fluorescent antibody test was carried out on slide and has sensitivity only 65%. The false positive and negative results chances are more in this test. Therefore, alone based on these test cannot be confirmed infection of H1N1 [35]. Rapid influenza antigen detection test was carried out by QuickVue kit using nasal swab (300 μL) but sensitivity was very poor 20%. Though, it is rapid but chances of false negative results are more [19,36]. Hemagglutinin inhibition (HI) test detects antibodies for which 8 units of HA is used as antigen. It is time consuming (20h) and not very specific [20]. Plastic based microfluidic chip detect antibody of H1N1 in blood by immunoreactions between GBP-recombinant influenza hemagglutinin antigen (GBP-H1a) fusion protein and fluorescent labeled antibody (Ab) by fluorescent technique. It is time consuming based on antigen-antibody interaction and detection range is 1.5 to 400 μg antibody mL^{-1} and better sensitivity achieved above μg antibody mL^{-1} concentration [23].

All available methods are time consuming, complicated, expensive, less sensitive (false positive or negative results) and mostly based on either antigen-antibody interaction or fluorescent based technique. The confirmation of disease cannot be ruled out based on single test. However, impedimetric DNA biosensor is highly specific based on HA gene

Fig. 5. Agarose gel (1.5%) electrophoresis of PCR product under UV illumination for confirmation of H1N1 samples. Marker: 50 bp DNA ladder, lane 1–6, 9 and 11 are showing approximately bands corresponding to 250 bp marker (H1N1 positive) and lane 7–8, 10, 12 and 13 are showing no bands (H1N1 negative).

Table 1
Comparison of different available methods for detection of swine flu (H1N1) in human.

No.	Methods	Target	Sensitivity (%)	Specificity (%)	Detection time[a]	References
1	Culturing of virus	Cytopathic effect	94	100	7–10 days	[33]
2	Real Time PCR	Swab	95	82	4–5 h	[33]
3	CF Test	Serum antibody	38	85	20 h	[20]
4	Immunodiffusion	NP, MP	–	–	7–10 days	[14]
5	ELISA	HA	57	100[b]	4–5 h	[34]
6	Direct Fluorescent-Antibody Test	Serum antibody	65	99	6–8 h	[35]
7	Rapid Influenza Detection Test	NP	20	99	1–2 h	[19,36]
8	HI Test	Serum antibody	91	25	18–20 h	[20]
9	Microfluidic immunosensor	Antibody-antigen interaction	–	–	5–6 h	[23]
10	Impedimetric sensor	cDNA ((HA Gene)	3750 Ω cm^{-2} ng^{-1} cDNA	100[c]	30 min	Present study

CF: Complement fixation; cDNA: Complementary DNA (Deoxyribonucleic acid); ELISA: Enzyme linked immunosorbent assay; HA: Hemagglutinin; HI: Hemagglutinin inhibition; NP: Nucleoprotein; MP: Matrix protein.
[a] Approx. detection time.
[b] Differ with virus load.
[c] Gene specific of H1N1.

sequence of H1N1 virus (100% homology of designed probe with DNA sequence of HA gene of H1N1 confirmed by Basic Local Alignment Search Tool [BLAST, Bioinformatics] for hybridization with target DNA of patient samples). The specificity was also confirmed by hybridization with non-specific pathogens. It requires small volume of DNA samples (6 μL) to cover the Au working electrode surface (0.126 cm^2). It is disposable screen printed Au electrode which can bind minimum (LOD) 0.004 ng DNA and requires only 6 μL volume to cover the working Au surface. Excess volume may cause spreading over other electrodes (counter and reference) causing wrong signals (results). Since, after hybridization with probe excess unbound DNA is washed out, the LOD of sensor cannot be changed even if made in higher concentrations (μg/mL or more). The LOD of sensor will remain 0.004 ng for this surface area (target DNA attached with probe after hybridization and cannot suspend further in liquid). The present sensor takes only 30 min for confirmation of the H1N1 infection and can be used simultaneously diagnosis of several patient samples using screen printed electrode array (multiple electrodes) during an outbreak of the disease for early diagnosis and medical care.

4. Conclusions

The H1N1 disease causes several deaths during an outbreak. The present available diagnostic techniques are either less sensitive or confirmation based on more than one test. Therefore, we focused to develop rapid, sensitive and non-expensive device to detect H1N1 during an outbreak to save life of several persons by using disposable cysteine modified screen printed Au electrodes. The fabricated impedimetric biosensor (HA gene specific immobilized probe) can detect as low as 0.004 ng ss-cDNA of H1N1 in 6 μL only in 30 min. The biosensor has sensitivity 3750 Ω cm^{-2} ng^{-1} DNA and highly specific to H1N1 virus and no other pathogens. The biosensor was also validated with real human patient samples of cDNA and correlated with already existing methods such as RT-PCR and results were found comparable. The hemagglutinin gene based H1N1 biosensor has good stability and can be used in remote areas where sophisticated instrument facility does not exist.

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Conflict of interest

The authors declare that there is no conflict of interest.

Appendix A. Supplementary data

Supplementary material associated with this article is available at https://doi.org/10.1016/j.ijbiomac.2019.02.149.

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