



Development of rapid colorimetric assay for the detection of Influenza A and B viruses

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ABSTRACT

The flu viruses are respiratory pathogens which, according to the World Health Organization (WHO), infect 5–10% of the world population resulting in 3–5 million cases of severe illness and 290,000 to 650,000 annual deaths. Early diagnosis and therapeutic intervention can ameliorate symptoms of infection and reduce mortality. The conventional diagnosis of viral infections, including flu viruses, has evolved over the years with diverse approaches, however, there are inherent short comings associated with these testing. There is an urgent need for rapid and low-cost diagnostic assays, due to the enormous annual burden of influenza diseases and its associated mortality.

In this study, novel, low cost and easy to use colorimetric flu virus biosensor assay was developed. The sandwich assay format was utilized using antibodies immobilized onto cotton swabs, for the rapid detection of flu A and B viruses. These swabs serve as sample collection, analytes pre-concentration as well as sensing tool. The proof of concept was established for this assay in buffer and mucus samples. The limit of detection (LOD) of the colorimetric assay was 0.04 ng mL⁻¹ for Flu A and Flu B respectively and with linear dynamic range between 0.04 ng mL⁻¹ to 40 ng mL⁻¹ for both viruses in mucous samples. The assay can be performed at the patient's bed side by minimally skilled hospital personnel without the need for instrumentation. Cross-reactivity assays testing was done using Flu viruses specific activated swabs reacted with other common respiratory viral pathogens' antigen, in order to assess the specificity of the swabs.

1. Introduction

Infectious diseases continue to be the bane of human well-being through the ages [1,2] and with the increasing application of technology to medical diagnostics, newer infectious agents are being identified while previously known pathogens are being better characterized and reclassified [3–5]. Although humans have been able to eradicate some infectious agents completely (e. g. smallpox) [6], other agents continue to be a threat to human health; one such agent is the influenza viruses (Flu viruses) [7]. Flu viruses are enveloped RNA viruses belonging to the *Orthomyxoviridae* viral family; they are sub classified into Influenza A, B, C, D viruses, the latter was recently identified in 2001 [8,9].

Orthomyxoviridae viruses produce two surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA) with the respective functions of attachment to susceptible host cells and release of progeny viral particles from infected cells [8]. Further subtyping of the flu viruses, particularly Flu A is based on the antigenic type of the HA and NA protein of which there are many [8]. Influenza A (Flu A), B (Flu B), and C (Flu C), viruses are known to cause human infections while influenza D virus (Flu D) is not very significant as a human pathogen [9,10]. The flu viruses are respiratory pathogens and according to the World Health Organization (WHO), they infect 5–10% of the world population resulting in 3–5 million cases of severe illness and 290,000 to 650,000 annual deaths [11]. Flu A and B viruses are known to produce

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homologous proteins due to genomic similarity, however, they have different promoter proteins responsible for transcription and replication as well as different accessory genes, these divergent proteins may assist in differential characterization of the two agents in the laboratory [12]. Flu A virus which has 8 segmented genome and diverse host range including humans, wild and domestic birds and pigs, exhibits antigen shift and drift. Antigenic shift results in the emergence of novel and occasionally deadly viral strains with pandemic potential while antigenic drift results in the emergence of slightly modified viruses with limited pandemic potential [8,12]. Flu B viruses do not undergo antigenic shift and as such do not cause pandemic infections [8]. These two flu viruses are however significant from the public health perspective.

The diagnosis of viral infections, including those due to flu viruses, has evolved over the years with diverse diagnostic approaches [13–17]. Methods such as viral isolation through conventional and shell culture, serology, various nucleic amplification techniques and more recently lab on a chip assays have been developed and applied in diagnostic [13,14,18–21]. Lab on a chip technology have demonstrated a good adaptation to point of care testing where in a patient's sample need not be sent to a central laboratory before therapeutic decision and/or infection control protocols are instituted as a consequence of rapid diagnostic result [19]. In view of the enormous annual burden of disease, associated mortality, and the issues associated with other conventional diagnostic methods such as long turnaround time for culture, high cost of reagents coupled with highly trained personnel required for molecular methods, a rapid diagnostic method for flu diagnosis is needed. Rapid diagnosis will lead to early and timely institution of antiviral therapy and early application of infection control protocols in a healthcare facility. These actions can ameliorate morbidity, reduce mortality and prevent the emergence of secondary cases when instituted early [17,22]. More researchers are now adapting nanotechnology to the rapid diagnosis of microbial infections including viruses, through biosensor devices [23–26]. The first biosensor was devised in the 1960s by Clark and Lyons [26] for the diagnosis of diabetes mellitus and these devices are increasingly being applied in the field of infectious disease diagnosis and screening as well as other fields such as the food industry, for rapid and low-cost detection of different analytes [16,20,23,24,27–30]. A biosensor, in its simplest definition, is an analytical device that converts a biological response into a readable signal. The readable signal is usually electrical in nature.

Electrochemical impedance immunosensors (EIS), a bio-affinity group sensor, have been used in the diagnosis and detection of flu viruses. EIS measure changes in surface conductivity associated with viral antigen detection through binding on the biosensor. Although EIS is a rapid assay when compared with viral culture, it has low marketability due to poor selectivity, time to detection and ability to monitor fluctuations in viral antigen binding [13]. Optical biosensors (OB) which are based on surface plasmon resonance phenomenon, have also been used in the detection of flu viruses. Inhibition assays were used to detect H1N1 and H3N1 subtypes of flu viruses under laboratory settings [13]. A major disadvantage of OB is that they are bulky and hard to miniaturize, making it difficult for field/point of care use.

Piezoelectric sensors were employed for the diagnosis of flu viruses. These assays measure the changes in the deflection of a microcantilever using deflector plates, resulting in the fabrication of sensitive biosensors; however, the major disadvantage of this assay is their sensitivity to environmental vibrations and fluctuations which make it difficult to be used in the field [13]. After careful consideration of the literature and commercially available products, there is a need to explore and develop low cost, easy to use point of care biosensor devices with high specificity and sensitivity for the detection of infectious agents such as the flu viruses.

In this study, we present a novel, low cost and easy to use flu viruses portable biosensing platform. The assay employs the sandwich assay format, for the rapid detection of Flu A and B viruses using recombinant antibodies immobilized onto cotton swabs as sample collection, pre-concentration and sensing tool. Finally, the cross-reactivity of the

antibodies functionalized swabs was evaluated using some other viruses' antigens.

2. Materials and methods

Flu A and B antigens and antibodies were obtained from Biospecific (Biospecific, Emeryville, CA, USA), while HCoV antigens and antibodies were obtained from Medix biochemica (Medix biochemica, Klovipellontie, Finland). MERS CoV antibody and antigen were obtained from Sino Biological Inc (Wayne, PA, USA). Carboxylic acid functionalized nanobeads of different colors (orange and blue) were obtained from Polysciences Inc. (Polysciences Inc., Warrington, PA). The blue beads were 300 nm spheres, while the orange beads were 200 nm in size.

All reagents were kept under manufacturer's specified conditions until use. Regular cotton swabs were bought from the local supermarkets. Linkers such as 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-Hydroxysuccinimide (NHS) were obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, USA). Phosphate buffer saline (PBS; pH 7.4) tablets, bovine serum albumin (BSA) were also obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, USA). All chemical reagents were laboratory grade.

2.1. Preparation of activated cotton swabs

The cotton swabs were activated by immersion in a mixture of 100 mL of 2 mM potassium periodate and 1 mL sulfuric acid overnight at room temperature (Scheme 1 (I)). Activation resulted in the conversion of polyhydroxyl groups present on the cotton swabs, to aldehyde groups, which can be used later for immobilization of the antibodies through the formation of amide bonds. The presence of the aldehyde group on activated swabs was confirmed using Fourier-transform infrared spectroscopy (FTIR). Activated swabs, were washed in order to remove excess oxidizing agent that might be present and thereafter kept in PBS buffer, until they were functionalized with antibody.

2.2. Immobilization of antibody on activated cotton swabs

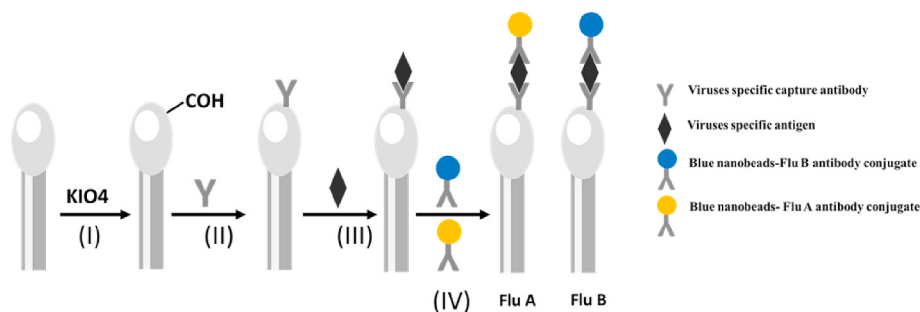
Immobilization of Flu A antibody on the swabs (Scheme 1 (II)) was achieved by incubating the activated swabs into a well-mixed solution of 40 μL of $2.2 \times 10^{-1} \text{ mg } \mu\text{L}^{-1}$ of Flu A antibody into 960 μL of PBS and incubated overnight at 4 °C. The swabs were rinsed to wash away any unbound antibody and thereafter 1% BSA (10 mg mL^{-1} distilled water) was added to block the active sites on the Q-tips. Forty micro liters ($1.1 \times 10^{-1} \text{ mg } \text{mL}^{-1}$) of Flu B virus antibody in 960 μL of PBS was immobilized onto the cotton wool, following the same method as stated above. Control swabs were incubated with 1% BSA solution in PBS buffer for 30 min at room temperature.

2.3. Immobilization of secondary antibodies on colored polymeric nanobeads

Three hundred microliters (300 μL) of the different colored polymer (nano) beads; orange for Flu A and blue for Flu B, was washed with distilled water 3 times before being reacted with a coupling solution of EDC/NHS; the beads were later washed in distilled water to remove excess coupling agent. Flu A antibody was coupled to activated orange polymer beads and Flu B antibody was coupled to activated blue polymer beads. Blocking of any unbound sites was achieved using 1% BSA solution in PBS for 30 min.

2.4. The assay procedure

The activated swabs with immobilized Flu A and B antibodies (Scheme 1 (II)) were reacted with their corresponding antigens (Scheme 1 (III)) for 3 min, in tubes containing 0.04 ng mL^{-1} , 0.4 ng mL^{-1} , 4 ng mL^{-1} and 40 ng mL^{-1} of Flu A and B respectively. Then, the swabs were



Scheme 1. Colorimetric assay for the detection of Influenza A and B viruses. (I) Activation of the Cotton swab; (II) Immobilization of Flu A or flu B on activated cotton swab; (IV) Capturing of flu virus corresponding antigen; (IV) Immobilization of colored nanobeads secondary antibody conjugate.

washed with PBS followed by immersion into 300 μ l solution of the activated colored polymer beads with immobilized secondary antibody (Scheme 1 (IV)). Reaction with the activated polymer beads was allowed to occur for 2 min. The swabs were washed to remove any unbound activated beads, results were inspected visually thereafter. Control assays were performed in parallel with each assay type. The assay duration was 5 min. Cross-reactivity assays were performed by reacting activated influenza antibodies bearing swabs against human corona virus (HCOV) and MERS Co V antigens.

developed at the National Institute of Health. In brief, a photograph of the cotton swab was captured following assay operation using a smartphone and saved in JPEG format. Then, image background was subtracted to avoid false-positive color selection. Adjusting color threshold by pass and stop brightness, saturation and Hue contrast provide distinguishable colored area. This segment area was selected and the color threshold measured. Photos analysis was performed at least three times on each cotton swab. Quantitative measurements are presented in Figs. 1–4 as mean $\pm$ 3 sigma deviation.

2.5. Quantitative detection

Quantitative detection was assessed using ImageJ program

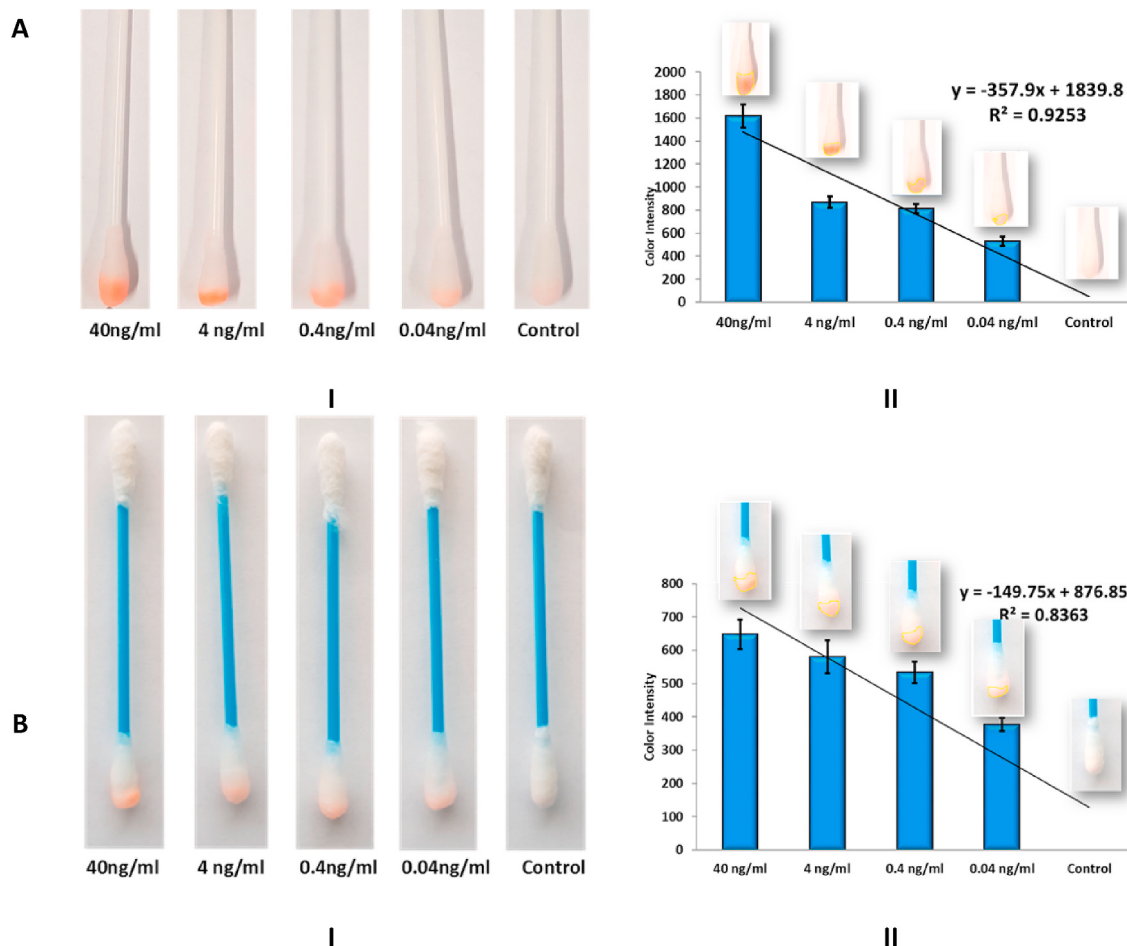


Fig. 1. Colorimetric assay for the detection of Flu A; (AI) in buffer and (BI) in mucus samples. Quantitative detection of Flu A as processed by ImageJ software (AII) in buffer; (BII) in mucus samples.

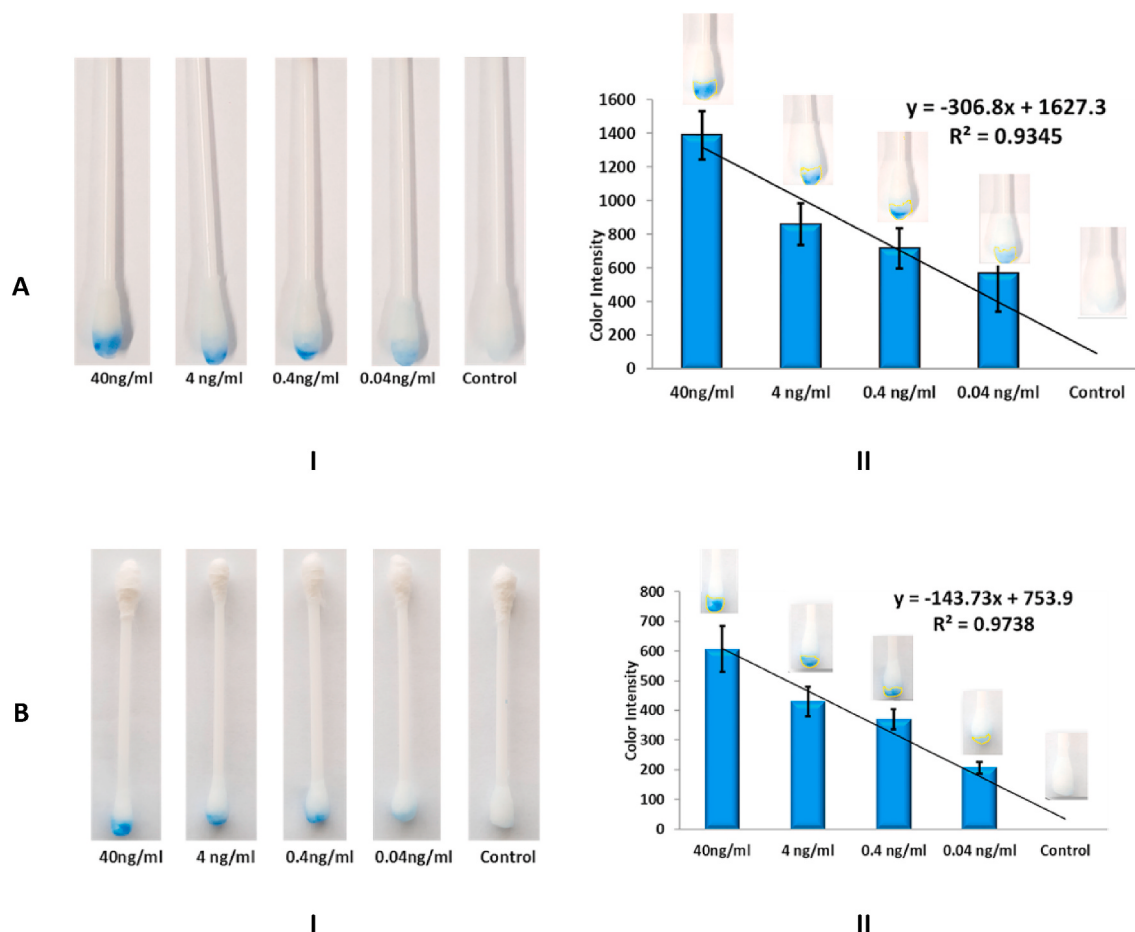


Fig. 2. Colorimetric assay for the detection of Flu B; (AI) in buffer and (BI) in mucus samples. Quantitative detection of Flu A as processed by ImageJ software (AII) in buffer; (BII) in mucus samples.

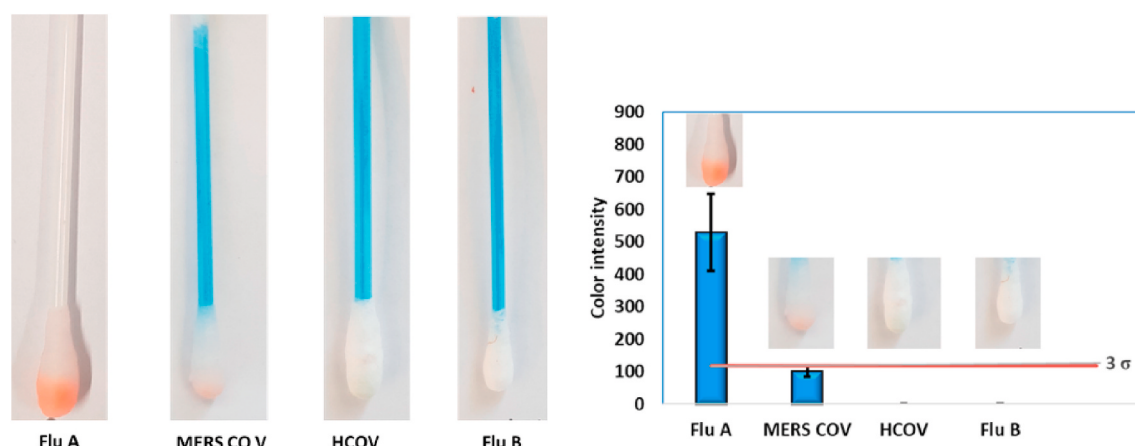


Fig. 3. Cross-reactivity of Flu A antibodies immobilized on swabs against HPV, MERS CO V, HCOV and Flu B antigens.

3. Results and discussion

3.1. Sensor preparation and characterization

Cotton is composed of cellulose, a macromolecule made up of long chains of glucose molecules connected by C-1 to C-4 glycosidic linkages. The hydroxyl groups present on the glucose monomers, were oxidized to aldehyde groups by reacting the cotton swabs with potassium periodate during the process of activation. Scheme 1 shows the various steps to

prepare the cotton swab and assay. Activated swabs were characterized using Fourier-transform infrared spectroscopy (FTIR), where a characteristic peak appears at 1730 cm^{-1} confirms the presence of aldehyde groups [31–33]. The activated swabs were then functionalized by immobilizing viruses specific capture antibodies; specifically, Flu A and B antibodies. These swabs captured the corresponding antigen of interest selectively, when applied to solutions of viruses or swabbed over artificially contaminated surfaces with the corresponding viral antigens. Thereafter, the swabs were immersed in functionalized colored

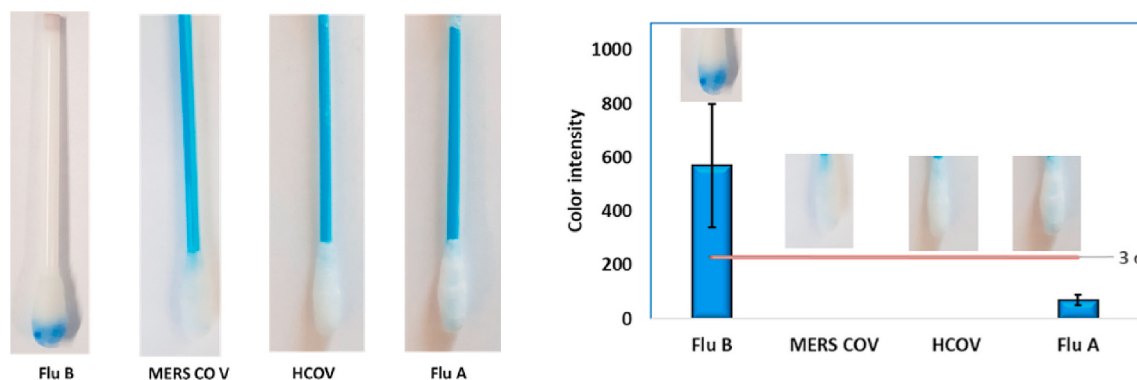


Fig. 4. Cross-reactivity of Flu B antibodies immobilized on swabs against HPV, MERS CO V, HCOV and Flu A antigens.

nanobeads solution. Orange nanobeads were functionalized with antibodies for Flu A and blue nanobeads for Flu B, the viral antigen was sandwiched between the capture antibody immobilized on the swab and the secondary antibody immobilized onto the nano beads. The reaction was visualized through the second step of the assay wherein activated nanobeads were retained on the applied functionalized swabs.

3.2. Assay development

Antibody functionalized swabs were demonstrated to bind specific antigens in the reaction mixture through visual inspection of the swabs after the binding of the immobilized colored nanobeads. Fig. 1A, B, 2A and 2B shows the qualitative and quantitative results of the proposed assay for different concentrations of Flu A and Flu B recombinant antigen in buffer and spiked mucus samples. Various surfaces were artificially contaminated with different concentrations of recombinant Flu A and Flu B antigens. The activated swabs were passed over the surfaces to collect the virus's antigens from the surfaces. Then, the cotton swabs washed with PBS and incubated in the colored beads functionalized with the secondary antibodies. The unbound beads were then removed by washing with buffer. Fig. 1A and B shows the gradual increase in the orange color with increasing the concentration of Flu A antigen. It is clear from Fig. 1A and B that the higher number of orange colored beads bonded to the high concentration of Flu A antigen. Fig. 2A and B shows similar trend for the detection of Flu B antigen swabbed on various surfaces artificially contaminated with various concentrations of Flu B using blue colored nanobeads. The limit of detection (LOD) of the colorimetric assay for Flu A was 0.04 ng mL^{-1} for Flu A and Flu B respectively. Nidzworski *et al.* employed boron-doped diamond electrochemical biosensor, and reported a limit of detection of 1 fg mL^{-1} of virus M1 biomarker in saliva buffer [34]. In the assay presented, proof of concept using recombinant viral proteins was achieved within minutes, unlike ELISA assay which can take few hours.

3.3. Cross-reactivity study

Specificity is one of the main criteria for evaluating the sensor's performance to be sure that the sensor is only selective for a specific antigen. A number of swab sensors were functionalized with antibody for Flu A and individually incubated with MERS CO V, HCOV and Flu B antigens, followed by incubation with orange colored nanobeads functionalized with antibodies for Flu A (Fig. 3) shows that there was no significant binding with the tested antigens and the Flu A sensors. Similarly, Flu B sensors were incubated with MERS CoV, HCoV and Flu A antigens, followed by immersion in the blue nanobeads solution functionalized with Flu B antibodies. Again, the biosensors did not reveal a positive reaction when it was applied to other non-specific antigens, indicating that non-specific adsorption on the sensors was insignificant. Cross-reactivity assays revealed the specificity of the functionalized

swabs even in the presence of other viral antigens contaminating the surfaces (Figs. 3 and 4). The lyophilized cotton swab sensors are stable for more than 6 months which is the time tested for so far at room temperature.

The assay takes an average of 5 min to perform and requires neither sophisticated equipment nor skilled personnel to perform, leading to reduction in cost and turnaround time. This low-cost and rapid approach as point of care testing and diagnosis can assist clinicians and their associated teams, to perform early interventional measures or implementing infection control protocols, that will prevent the spread of flu viruses in a healthcare facility. With the established proof of concept, further studies aimed at multiplexing the assay has begun, this will allow for a low cost, rapid screening of multiple pathogens at first contact with the patient.

4. Conclusion

In this study, simple, easy to use colorimetric, instrument-free assay was developed for the detection of Flu A and B viruses. The diagnostic tool employed cotton swab biosensor for sample collection, pre-concentration as well as sensing element. Sandwich immunoassay principle was employed for the detection of the flu antigens using various colored nanobeads. The assay displayed good sensitivity and selectivity for the Flu viruses' antigens tested. Although, a proof of concept in this study was demonstrated, further studies aimed at multiplexing and field application in clinical samples should be evaluated. We would like to conclude that the assay is simple, not expensive, portable and user-friendly, with a high potential for adaptability for field work and point of care testing, especially in resource limited settings.

Authors credits

Muhabat Adeola Raji and Yumna Alorajj did most of the experimental work. Fatimah Alhamlan performed some testing. Ghadeer Suaifan proofed reading and performed the quantitative data. Karina Weber, Dana Cialla-May, Jürgen Popp and Mohammed Zourob supervised the work and proof read the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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