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Development of a Rapid Immuno-Based Screening Assay for the Detection of Adenovirus in Eye Infections

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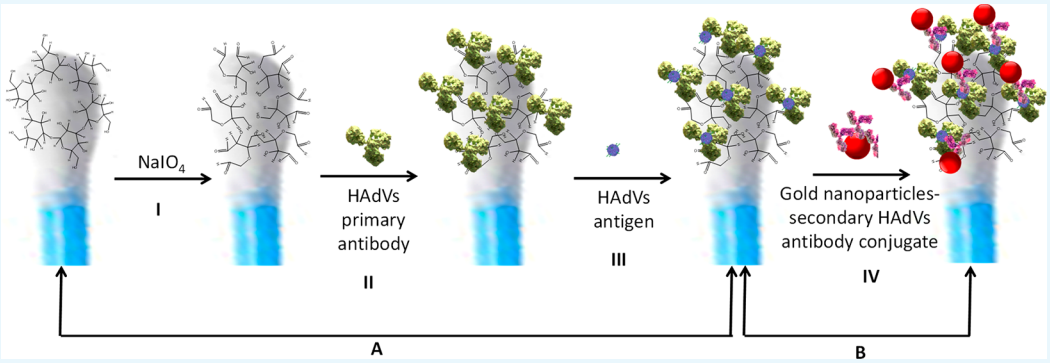

ABSTRACT: Despite progress in fighting infectious diseases, human pathogenesis and death caused by infectious diseases remain relatively high worldwide exceeding that of cancer and cardiovascular diseases. Human adenovirus (HAdV) infects cells of the upper respiratory tract causing flu-like symptoms that are accompanied by pain and inflammation. Diagnosis of HAdV is commonly achieved by conventional methods such as viral cultures, immunoassays, and polymerase chain reaction (PCR) techniques. However, there are a variety of problems with conventional methods including slow isolation and propagation, inhibition by neutralizing antibodies, low sensitivity of immunoassays, and the diversity of HAdV strains for the PCR technique. Herein, we report the development and evaluation of a novel, simple, and reliable nanobased immunosensing technique for the rapid detection of human adenoviruses (HAdVs) that cause eye infections. This rapid and low-cost assay can be used for screening and quantitative tests with a detection limit of 10^2 pfu/mL in less than 2 min. The sensing platform is based on a sandwich assay that can detect HAdVs visually by a color change. Sensor specificity was demonstrated using other common viral antigens, including Flu A, Flu B, coronavirus (COV), and Middle East respiratory syndrome coronavirus (MERS COV). This cotton-based testing device potentially exhibits many of the desired characteristics of a suitable point-of-care and portable test, which can be carried out by nurses or clinicians especially for low-resource settings.

INTRODUCTION

In spite of the remarkable technological advances in sanitation and the effort to identify, monitor, and control infectious diseases including those of viral origin, viruses remain the cause of the marked increase in human pathogenesis and death throughout the world exceeding cancer and cardiovascular illnesses.[1] The human adenovirus (HAdV) group is part of the Adenoviridae family. This name originated from the adenoid tissue cell culture from where these viruses were first isolated. To date, over 52 different serotypes have been described.[2] HAdV predominantly attacks cells of the upper respiratory tract[3] causing flu-like symptoms such as coughing and runny nose accompanied by pain and inflammation. In humans, adenovirus infections spread from one individual to another through droplets of ocular or respiratory secretions.[4] Accordingly, different HAdVs can lead to epidemic kerato-conjunctivitis (EKC) and acute follicular conjunctivitis (AFC).[5−8] Almost 92% of eye infections are thought to be associated with adenovirus. In healthy eyes, most infections are mild and self-limiting, whereas in compromised surface conditions vision-threatening sequelae may result.[9−11] Thus, speedy and accurate diagnoses of eye infections are an integral part of disease management in immunocompromised patients and children.[12−18]

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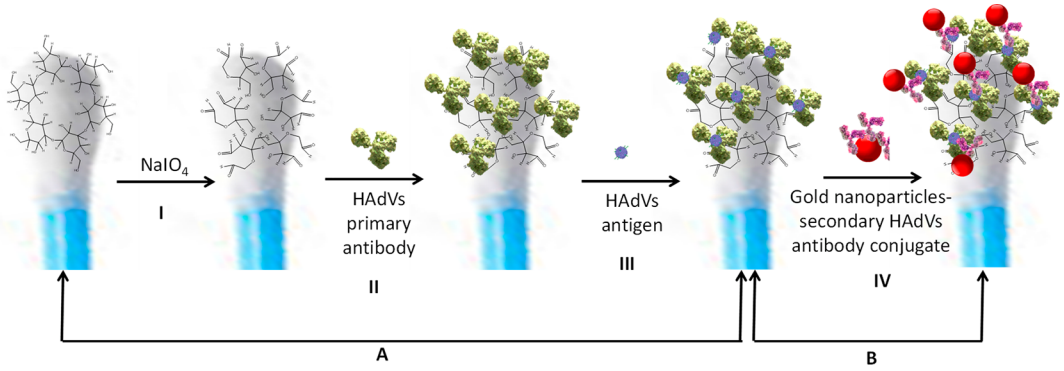
Scheme 1. Schematic Diagram of the HAdVs Colorimetric Sandwich Immunosensing Assay[a]



[a](A) Preparation of a cotton swab−primary antibody sensor. (I) Oxidative activation of the cotton swab. (II) Primary antibody immobilization on the cotton swab surface. (III) Capturing of HAdVs antigen. (B) Colorimetric detection assay (IV) HAdVs antigen sandwiched between the cotton-immobilized primary antibodies on the sensor and secondary detection gold nanoparticle−antibody conjugate.

HAdV diagnosis is commonly achieved by conventional assays such as viral cultures which detect HAdVs associated with respiratory and/or systemic infections. This gold standard method is sensitive for many serotypes; yet, virus isolation and propagation are slow and can be inhibited by neutralizing antibodies and other interfering substances. Alternate direct antigen detection assays such as immunofluorescence, radioimmunoassay, or enzyme-linked immunosorbent assay (ELISA) identified respiratory and gastrointestinal HAdVs with acceptable specificity and a fast turn-around time but were less sensitive than the culture method. Additional assays such as electron microscopy detected gastroenteric HAdV serotypes in stool samples but was not very sensitive.[19] Currently, the polymerase chain reaction (PCR) technique based on the amplification of viral DNA directly from patient specimens serves as a front-line diagnostic procedure due to fast turn-around time and good sensitivity. However, this method is challenged by the diversity of HAdV species.[20] Several commercial qualitative and quantitative nucleic acid amplification tests (NAATs) are available, but the costs for these tests are unaffordable for many laboratories. Following the need for fast, selective, stable, and low-cost diagnostic methods, different biosensor technologies were established. For example, research work on viral biosensing using electrochemical transduction, typically using antibodies, oligonucleotides, or aptamers as a recognition element, has been developed. These assays have the advantage of being low-cost, robust, and relatively simple to operate, require minimal preparative steps, and work directly in biological matrices such as serum, milk, and urine.[21−24] Other detection techniques include the use of gold nanoparticle (AuNP) *in vitro* assays for virus detection. These assays represent a promising approach due to AuNPs' unique physical properties,[25] excellent optical performance, and special catalytic activity coupled with their molecular interaction specificity with various biomolecules (e.g., antibodies, single-stranded (ss) DNA, and RNA aptamers).[26−28] These assays are characterized by their sensitivity and applicability for the quantitative detection of viruses with excellent multiplexing capabilities.[29,30] However, the LOD of AuNP-based assays is dependent on AuNPs size (a few to tens nanometers) and shapes (e.g., sphere, rod, core−shell, cube, star, cage, pyramid, Janus, etc.).[31] which is associated with their physical and optical properties. However, gold nanoparticle (AuNP)-based assays have great potential for urgent unmet biomedical needs.

It is a common practice for a patient sample to be analyzed in laboratories by a conventional method such as tissue culture, polymerase chain reaction (PCR), and enzyme-linked immunosorbent assays (ELISA). However, all these methods need to be performed in centralized laboratories and require skilled personnel and sample preparation. Viruses detection by molecular testing is based on samples sent to the lab, which is lengthy process and might pose a risk of contamination or damage during transportation and/or processing steps. Accordingly, optimum sample collection, transportation, and processing techniques are crucial. Several sample collection tools have been reported to reduce the risk of contamination in laboratories while running the traditional methods for virus detection. These methods include dried blood spot collection onto filter paper,[32,33] use of FTA filter paper impregnated with lyophilized chemicals to lyse both viruses and bacteria rendering them noninfectious,[34] swabs fixed in ethanol to collect respiratory samples for surveillance purposes,[35] and dry swabs broken into the lysis buffer on receipt to the laboratory.[36] These collection techniques are guaranteed to maintain viral RNA integrity for long periods and at various temperatures. Interestingly, dry cotton-tipped wooden-ended swabs present a simple, cheap, and convenient collection tool that is widely available in all clinical wards.

Currently, there are no diagnostic tests on the market that can detect adenovirus *in situ* from patient tears. In this work, we present the development of a novel, cotton swab nanobased immunosensing screening assay for the detection of adenovirus infection in the eyes. As shown in Scheme 1, this sandwich-type assay is based on the use of an adenovirus primary antibody-immobilized cotton swab as capture probe and the use of gold nanoparticle conjugated secondary antibodies as a signal probe. In this onsite assay, cotton-tipped ended swabs will act as a collection, preconcentration, and detection tool, and there will be no need for virus sample processing, which in turn will reduce assay associated costs, storage requirements, and the risk of leakage/damage/contamination during transportation.

RESULTS AND DISCUSSION

Sensor Preparation and Characterization. Cotton as a natural cellulose has many ideal characteristics such as their

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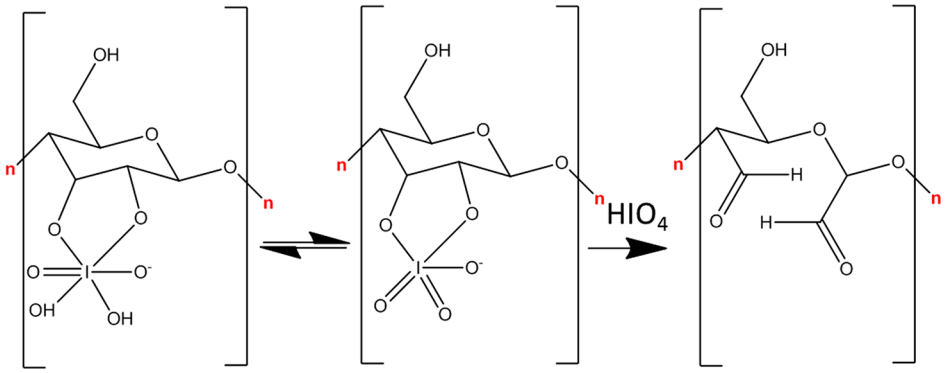
Scheme 2. Mechanism of Cellulose Oxidation



wide use in the biomedical sector in diagnostics and therapy, good absorbency, and good color retention ability. Moreover, following cellulose oxidation, the functional groups formed can be used for immobilization of biomolecules. For example, when a cellulose oxidation reaction is carried out using periodate ions (IO_4^-), a break in the glucopiranosic cycle between C2 and C3 results in the formation of dialdehyde cellulose as shown in Scheme 2.[25,26,37,38] In this study, HAdVs have been optically screened using a sandwich immunoassay method, in which the virus is sandwiched between a primary antibody immobilized over a cotton swab surface, and a secondary antibody conjugated with gold nanoparticles as shown in Figure 1. In this nanobased colorimetric sandwich

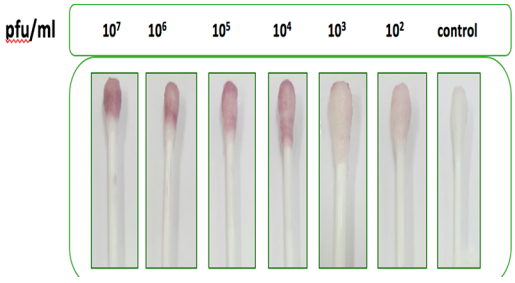


Figure 1. Detection of different concentrations (10^2–10^7 pfu/mL) of HAdVs antigen in spiked artificial tears; the last cotton swab represents a control containing no virus.

immunoassay, a cotton swab act as a supporting matrix, the primary antibody as a capturing agent, and secondary antibody-conjugated gold nanoparticles for the detection.

The activated cotton swabs were functionalized by immobilizing HAdV-specific capture antibodies over the surface. Then, the swabs were applied to 10-fold serial dilutions solutions (10^2–10^7 pfu/mL) of HAdV antigen as shown in Figure 1. Thereafter, the swabs were immersed in a specific secondary antibody-conjugated gold nanoparticle solution. The intensity of the color observed was directly proportional to the concentration of the virus captured as more adenovirus sandwich complexes are formed at higher concentrations. A control sample was prepared by adding distilled water instead of HAdVs antigen so that a difference in color is observed.

Figure 2 shows the calibration curve using nanobased sandwich colorimetric immunoassays for different HAdV concentrations constructed by plotting the concentration versus the color intensity as calculated by image J software.[39−45] It is clear from Figure 2 that the signal increases

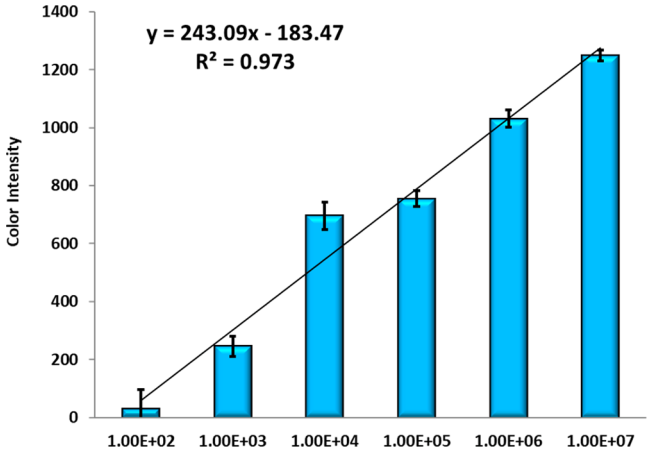


Figure 2. Calibration curves of different HAdV concentrations using the sandwich colorimetric immunoassay; a plot of color intensity versus HAdV concentration.

with increasing the viral loads. This is very useful in determining roughly the virus load in infection and can be used for monitoring the efficiency of treatment. The current rapid colorimetric sensor gave a low detection limit in a short analysis time. Table 1 shows the different methods used for the detection of adenoviruses their advantages and disadvantages.

Specificity Test. Specificity is an indicator of the accuracy and success of the sensor performance. In other words, a successful sensor yields positive results for the target virus only. Specific binding of adenovirus antibody has been tested using four different viruses such as Flu A, Flu B, COV, and MERS COV antigens. Four cotton swabs conjugated with adenovirus antibodies were used for individually swabbing different samples of Flu A, Flu B, COV, and MERS COV antigens. Each cotton swab was further incubated with a developing solution containing gold nanoparticles conjugated with adenovirus secondary antibodyies for 3 min. After extensive washing with PBS, none of the cotton swabs displayed a significant red color of gold nanoparticles over the cotton surface (Figure 3).

Real Samples Testing. Environmental contamination with adenovirus is a common source of infection, and eye clinics present a fertile ground for outbreaks. Adenovirus is known to infect the ocular surface and cause adenoviral conjunctivitis. This infection is quite contagious, as the virus is transmitted readily in patient ocular secretions, contaminated fomites (including eye droppers and mascara bottles), and even contaminated swimming pools.[46] Therefore, biosensing swabs

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Table 1. Adenovirus Detection Methods and Their Advantages and Disadvantages

detection method	limit of detection	assay time	advantages	disadvantages	ref
electron microscopy			-Simple and rapid	-Low sensitivity and specificity	47
			-No prior knowledge of virus is required	-Analysis based on morphology only	
			-No reagent selection is required	-Expensive equipment	
				-Expensive maintenance	
				-Experienced technician	
				-Not suitable for large numbers	
conventional plaque assay		require incubation times of seven or more days	-Sensitive for many serotypes	-Time consuming (lasting up to 14 days) and operator error-prone	48
conventional enzyme-linked immunosorbent assay (ELISA) methods	10^2 $TCID_{50}$ in a four-day test and 10^3 $TCID_{50}$ in the two-day assay	days	-Most common	-Time-consuming	49, 50
			-Relies on complex enzyme labeling methodology and specialized reagents	-Lack of sensitivity	
			-Relies on skillful operators and expensive instruments	-Specific	
real-time PCR assays	2.60 to 9 log 10 copies/mL	days	-Fast turn-around time with good sensitivity *Allowed for a rapid semiquantification	-Require specific primers, extraction, and purification of nucleic acids from preconcentrated samples	51, 52
				-Require specialized reagents such as DNA-binding dyes and fluorescent probes	
				-Require expensive instrumentation	
				-Application in limited resources is not feasible	
multiplex PCR-enzyme hybridization assay (Adenoplex)	100 and <1000 copies of DNA/mL	assay completed within 5 h	-Rapid, specific, and sensitive	-Demands pretreatment of samples and use of expensive antibodies or requires virus modification	53
			-Able to differentiate between HAdV species		
nested PCR assays	400 and 2500 copies/mL and 640 copies/mL	hours	-Good sensitivity	-Involve a two-step PCR procedure, increasing the time to complete the assay and the chance of carry over contamination	54, 55
			-Cost effective in a routine laboratory		
flow cytometry-based protocols				-Require labeled virus. *Viral titer estimation is influenced by culture working volume, duration of the assay, size of target cells	56−58
cell-based fluorescent biosensor	10^5 infectious adenovirus particle/mL	two days post infection	-Fast, easy, and label-free detection assay	-Requires expensive instrumentation	
				-Application in limited resources is not feasible	
nanobased immunosensing biosensor	10^2 pfu/mL	<2 min	-Speedy as it takes an average of 5 min to perform		current study
			-Suitable for point-of-care application, simple, low priced		
			-Does not require sophisticated equipment nor skilled personnel to perform		
			-Low-cost as point-of-care testing and diagnosis		

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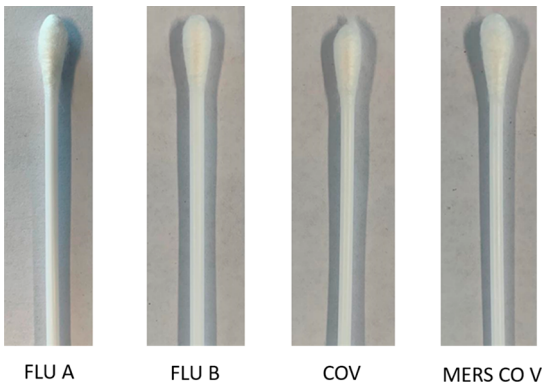

Figure 3. Specificity of adenovirus antibodies immobilized on cotton swab against Flu A, Flu B, COV, and MERS COV antigens.

developed were utilized to test eye infections in mice as an animal model following contamination by adenovirus as a function of time. Figure 4 shows the results of the Q-tips swab assay collected from the eyes of infected mice after 2, 4, 6, 8, and 12 days from the day of the infection. It is clear from Figure 4 that the intensity of the red color increases as a function of time due to the increase in the virus load in the infected eyes of the mice.

CONCLUSIONS

In conclusion, we developed a simple, low-cost, rapid, reliable, and portable-detection nanobased biosensing screening assay for the detection of eye infections caused by adenovirus. In this assay, a specific adenovirus primary antibody-immobilized cotton swab is used for preconcentrating the virus followed by complexing with gold nanoparticles conjugated to a secondary antibody for detection. The color of detection was red due to the attachment of the gold nanoparticles. The color was directly proportional to the concentration of the adenovirus captured on the cotton swab. The assay is simple and does not require any instrumentations, and the virus can be rapidly traced visually by the naked eye. The specificity of the assay was evaluated by treatment with different types of viruses. This rapid and low-cost assay can have enormous applications in clinics, hospitals, and many other biomedical fields. Cotton-based devices potentially exhibit many of the desired characteristics of suitable point-of-care viral testing. These diagnostic tests are inexpensive, portable, and simple to operate by nurses and clinicians at the doctor's office or in the field, making them appropriate for isolated places and less-resourced areas.

EXPERIMENTAL SECTION

Materials. Flu A and Flu B antigens were obtained from Biospacific (Biospacific, Emeryville, CA, USA), while coronavirus (CoV) and MERS COV antigens and antibodies were obtained from Medix biochemica (Medix biochemica, Klovinpellontie, Finland). Adenovirus was provided by Dr. Fatemah Alhamlan from King Faisal Specialist Hospital and Research Center (KFSHRC) in Riyadh, KSA. Gold nanoparticles were obtained from Atlas Medical Company (Amman, Jordan). All reagents were stored under the manufacturer's specified conditions until use. Regular cotton swabs were purchased from a local pharmacy in Riyadh, Saudi Arabia. Sodium periodate ($NaIO_4$), bovine serum albumin (BSA), 1-ethyl (3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and *N*-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, USA). Phosphate buffer saline (PBS; pH 7.4) tablets were obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, USA). All chemical reagents were laboratory grade.

Preparation of Activated Cotton Swabs. Natural cellulose fiber, cotton, with polyhydroxyl groups can be oxidized to aldehyde functional groups offering an approach for immobilizing biomolecules such as antibodies. In this study, cotton swabs were oxidatively activated by immersing them in a mixture of 2 mM $NaIO_4$ (100 mL) and aqueous concentrated H_2SO_4 (1 mL) for 16 h at room temperature (Scheme 1A–I). Cotton swabs were then washed thoroughly with cold water to remove the excess oxidizing agent. FTIR

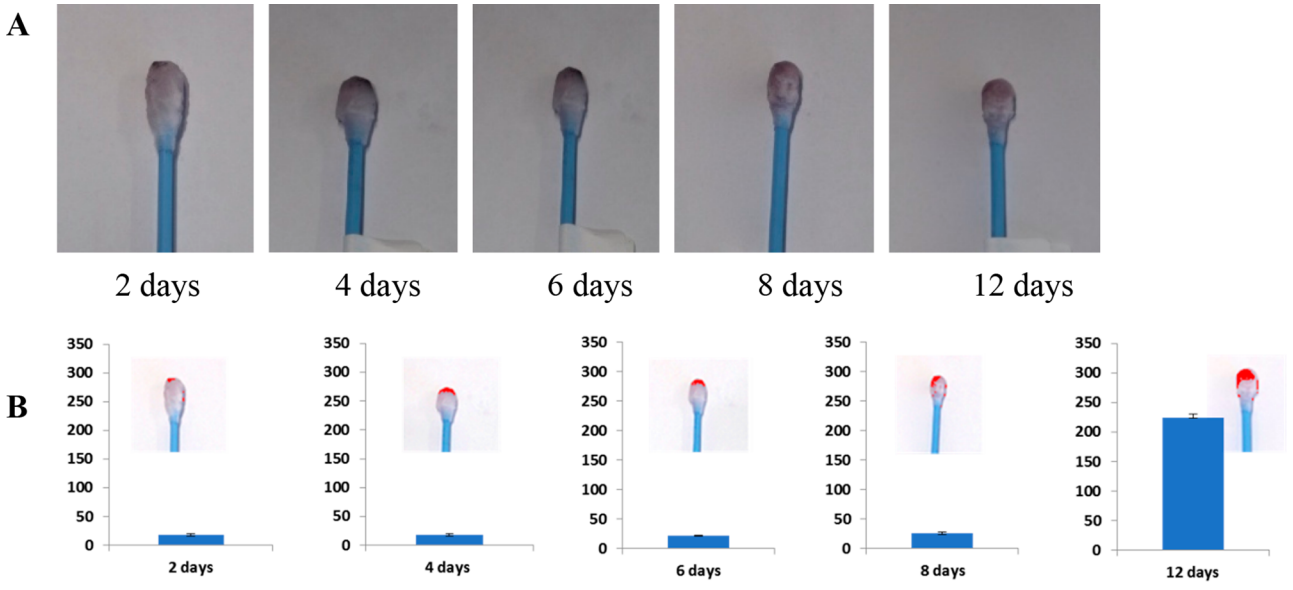

Figure 4. (A) Detection of the adenovirus virus in the eyes of infected mice after 2, 4, 6, 8, and 12 days from the administration of adenovirus into the eye of the mice; (B) quantitative load of the virus collected by the swab after 2, 4, 6, 8, and 12 days after infection.

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was applied to confirm cellulose hydroxyl groups' oxidation into aldehyde.[44] Activated cotton swabs were stored for later use.

Immobilization of Antibody on Activated Cotton Swabs. Immobilization of adenovirus primary antibodies onto the cotton swabs surface (Scheme 1A-II) was achieved by incubating activated swabs in a well-mixed solution of 40 μL of 2.2×10^{-1} mg/mL of adenovirus antibody into 960 μL of PBS (pH 7.4) for 18 h at 4 °C. Antibody-conjugated cotton swabs were rinsed with PBS (pH 7.4) to remove unbound antibodies and thereafter excess activated aldehyde groups were blocked by incubating the cotton swabs in 1% BSA (10 mg/mL distilled water) for 30 min at room temperature, followed by washing with PBS buffer (pH 7.4) three times. The antibody-conjugated cotton swabs were stored at 4 °C in PBS (pH 7.4) for further use. Control swabs were incubated with 1% BSA solution in the same manner.

Immobilization of the Secondary Antibodies on the Gold Nanoparticles. A 300 μL gold nanoparticle (6.7×10^{12} /mL, 30 nm in diameter) suspension was centrifuged at 16000−18000 rpm for 10 min. Collected pellet was resuspended in water and washed three times with water (1 mL) before being mixed with the secondary adenovirus antibodies (20 μL) for 2 h at room temperature. Finally, 1 mg/mL of BSA was added to block the unreacted active sites on the antibody−gold nanoparticles conjugate (Scheme 1B−IV).

Colorimetric Assay. The colorimetric assay consists of two steps;: the first step (Scheme 1A) involves virus capturing by wiping an activated adenovirus primary antibody bearing swabs over surfaces contaminated with 10-fold serial dilutions of the virus. Thereafter, the cotton swab-immobilized primary antibody−virus complex was washed twice with PBS buffer (pH 7.4) to remove the unbound virus antigen from the cotton. The second step (Scheme 1B) comprises a detection process, in which adenovirus antigen is sandwiched between the cotton-immobilized primary antibody on the sensor and a secondary detection antibody conjugated with gold nanoparticles for the color development as shown in Scheme 1. In this detection step, the cotton swab−primary antibody−virus complex was immersed in a solution of gold nanoparticles linked with a secondary antibody in PBS buffer solution for 3 min. The cotton swab then washed twice with PBS buffer to remove the unbound nanoparticles. Development of the red color indicates the attachment of the gold nanoparticles-conjugated antibodies to the captured virus collected on the cotton swab (Scheme 1B−IV). The experiment was repeated three times. Control assays were performed in parallel. The assay duration was 5 min. Cross reactivity tests were performed by reacting activated adenovirus antibodies bearing swabs against coronavirus (COV) and MERS COV, Flu A, and Flu B viruses.

Quantitative Detection. The developed assay was intended for visual observation of the color change over the cotton swab from white to red via the naked eye. The intensity of the color on the cotton swabs surface was directly proportional to adenovirus concentrations tested (10^2−10^7 pfu/mL). Following colorimetric assay operation, cotton swabs images were taken by direct photography using a smartphone camera and saved in JPEG format. For quantitative measurements, the intensity of the color was determined by using the ImageJ program (a freely downloadable program that can be used on any computer with Java 5 or a virtual machine) developed at the National Institutes of Health. Using the RGB stack command of ImageJ, each photo was split into red, green, and blue channels. Images were processed through the red channel, having lower background levels to avoid false-positive color selection. The colored area was highlighted manually by the color threshold function, and then the area was measured. However, as the threshold adjustment process is subjective, two different individuals performed the analysis by applying a similar protocol. To gain insight into quantitative measurement reliability, photo analysis was performed at least three times on each cotton swab. Quantitative measurements are presented in Figure 2 as mean ± RSD. Coefficient of variation (CV) was calculated and was found to be ≤1, illustrating a low variance in data distribution.

Testing of Adenovirus−Related Infections in Animal Modesl. The clinical applicability of sandwich biosensing assay has been validated by examining adenovirus-related infections *in situ* in infected mice eyes. Wild type (WT) C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME USA) at 6−10 weeks of age and 16−22 g weight. All mice were housed under specific pathogen-free conditions with a 12-h light/dark cycle and free access to food and water. Fifty micromolar normal saline contains 10^3 pfu/mL adenovirus was administered as drops in the eyes of the mice. As controls, mice were administered 50 μL of normal saline in the eyes. The animal study was carried out according to a protocol approved by the Animal Care and Use Committee. Swabbed samples were collected every 2 days and examined using the designed assay. The experiments were conducted in triplicate.

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Notes

The authors declare no competing financial interest.

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