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# A rapid detection method for Vaccinia virus, the surrogate for smallpox virus

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## Abstract

Prior to the World Health Organization's announcement of total eradication in 1977 [J. Am. Med. Assoc. 281 (1999) 1735], smallpox was a worldwide pathogen. Vaccinations were ceased in 1980 and now with a largely unprotected world population, smallpox is considered the ideal biowarfare agent [Antiviral Res. 57 (2002) 1]. Infection normally occurs after implantation of the virus on the oropharyngeal or respiratory mucosa [J. Am. Med. Assoc. 281 (1999) 2127]. Smallpox virus can be detected from the throats of exposed individuals prior to onset of illness and prior to the infectious stage of the illness. A rapid, sensitive real-time assay to detect Variola virus (smallpox) has been developed using the Vaccinia virus, a surrogate of smallpox, as a target. Cyanine 5 dye-labeled anti-Vaccinia antibody was used in a sandwich immunoassay to produce a fluorescent signal in the presence of the Vaccinia virus. The signal was detected using the Analyte 2000 biosensor (Research International, Monroe, WA). The Analyte 2000 uses a 635 nm laser diode to provide excitation light that is launched into a polystyrene optical waveguide. Fluorescent molecules within the evanescent wave are excited and a portion of their emission energy recouples into the waveguide. A photodiode quantifies the emission light at wavelengths between 670 and 710 nm. The biosensor was able to detect a minimum of  $2.5 \times 10^5$  pfu/ml of Vaccinia virus in seeded throat culture swab specimens.

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## 1. Introduction

Smallpox or Variola, a member of the genus *Orthopoxvirus*, is a large brick-shaped DNA virus with a diameter of about 200 nm (Fenner et al., 1988b). Smallpox has a fatality rate of 30% or more among unvaccinated persons; currently there is no known cure (Fenner et al., 1988a). The virus has a small infectious dose and is stable in an aerosol form; therefore, an aerosol release could cause a widespread outbreak (Harper, 1961; Wehrle et al., 1970). Although the disease smallpox has been eradicated worldwide, it is a major threat as a bioterrorism agent. It was probably first used as a biological weapon by the British during the French and Indian Wars in the mid-1700s. Contaminated blankets were distributed to the Indians to initiate outbreaks of the disease. The ensuing epidemics killed over 50% of the af-

ected tribes (Bremner and Henderson, 1988). In 1980, the same year that vaccination against *Variola major* (smallpox) ceased globally, the Soviet government began research to grow large quantities and adapt it for use in bombs and intercontinental ballistic missiles (Alibek, 1999). Today, with a lack of vaccinations, the long incubation period of the smallpox virus, and our rapid transportation capabilities, an outbreak could easily spread throughout the world. The recent outbreak of severe acute respiratory syndrome (SARS) is an example of such rapid worldwide dissemination. Variola virus is considered a Category A Pathogen by the National Institute of Allergy and Infectious Diseases (NIAID), meriting this ranking because of its high case-fatality rate and transmissibility.

Variola virus infection normally occurs after primary implantation of the virus on the oropharyngeal or respiratory mucosa, spreading from person to person by droplet nuclei, by aerosols expelled from the oropharynx or by direct contact (Henderson et al., 1999). By sampling individuals that had household contact with smallpox victims, Sakar et al.

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(1974) were able to detect smallpox virus from the throats of these individuals prior to the actual onset of illness and the infectious stage. The virus typically has a 12–14-day incubation period before the victim may experience any symptoms such as high fever, headache, abdominal pain, and delirium. The distinctive rash generally does not develop until 3–5 days following the prodromal stage.

A person immunized prior to exposure is assumed to be fully protected. Data on post exposure is sparse, but it is indicative of partial protection when vaccination occurs within 4 days of exposure (Mortimer, 2003). If the disease state does occur after a post exposure vaccine (given within 4 days), a reduction in severity is noticeable. Persons given the vaccine more than 4 days after exposure to the disease had a high incidence of severe and sometimes fatal smallpox. Unfortunately, because of the long incubation period, people may not realize they have been exposed in time for the vaccine to be effective. A rapid non-labor intensive method to detect Variola virus from patients' throat swab specimens could be used as field-based biological defense to prevent a pandemic if Variola virus were ever released by aerosol during a bioterrorism event.

Currently, in a pre-event setting if a patient has an acute onset of fever, >101 °F followed by a rash characterized by vesicles or firm pustules all in the same stage of development, confirmatory laboratory tests are run in a CDC Laboratory Response Network Level C or D laboratory. These tests include PCR identification of Variola DNA or negative stain electron microscopy (EM) identification of Variola virus. Confirmed smallpox victims might not receive smallpox vaccine until well after the 4-day window.

This paper describes the use of an evanescent wave, fiber optic biosensor to rapidly detect Vaccinia virus. The Analyte 2000 evanescent wave, fiber optic biosensor, developed at the Center for Bio/Molecular Science and Engineering, Naval Research Laboratory (Anderson et al., 1996) has been used to detect various analytes, including staphylococcal enterotoxin B (Templemen et al., 1996), *Escherichia coli* O157:H7 (DeMarco et al., 1999; DeMarco and Lim, 2001; DeMarco and Lim, 2002), cholera toxin (Bedenbaugh, 2002), and *Bacillus anthracis* spores (Lim, 2003). Rapid biosensor detection of smallpox virus from patients' throat swab specimens could reduce morbidity and mortality in a bioterrorism-related outbreak.

## 2. Materials and methods

### 2.1. Virus

Cobalt-irradiated Vaccinia virus (Lister strain), Osbourne Scientific Lot # 1L0004, was obtained through the Joint Program Office for Biological Defense (JPO-BD, Dugway, UT). Serial dilutions of this viral stock were made in sterile phosphate buffered saline (PBS) for biosensor assays.

### 2.2. Antibodies and labeling

Rabbit polyclonal anti-Vaccinia antibody was obtained from JPO-BD at a concentration of 6 mg/ml in 10 mM PBS 0.05% azide. The FluoroLink™Cy5 Reactive Dye pack (Amersham Life Sciences, Arlington Heights, IL) was used to label the detection antibody with Cy5. Antibodies were diluted to 2 mg/ml in 0.1 M carbonate–bicarbonate buffer (pH 9.3), 500 µl total. The entire 500 µl was added to the dye vial, capped, mixed thoroughly, and incubated in the dark at 25 °C for 30 min. Labeled antibody was purified from free dye by gel filtration on a Bio-Gel P10 (Bio-Rad, Hercules, CA), exclusion limit 1500–20,000 Da, equilibrated with PBS, 0.02% sodium azide. Fractions containing labeled antibody were collected and analyzed spectrophotometrically on a DU®-64 spectrophotometer (Beckman, Fullerton, CA). Dye to protein ratios were calculated and ranged from 0.9 to 1.1. Stock solutions of Cy5 labeled anti-Vaccinia antibody were stored in the dark at 4 °C until needed. Biotin labeling of the antibody was carried out according to manufacturer's directions. Antibodies were diluted to 2 mg/ml in carbonate buffer (pH 8.5). After 0.5 mg of EZ link NHS-LC-LC Biotin (Pierce Biotechnology, Rockford, IL) was dissolved in 1 ml *N,N*-dimethylformamide (DMF), 75 µl of this solution was added to 475 µl of antibody solution, inverted several times to mix and incubated on ice for 2 h. Unincorporated biotin was removed by gel filtration on a Bio-Gel P10 equilibrated with PBS 0.02% sodium azide. Stock solutions of the biotin labeled anti-Vaccinia antibody were stored at 4 °C until needed.

### 2.3. Enzyme-linked immunosorbent assay (ELISA)

ELISAs were initially performed as part of the biosensor assay development. Volumes of 100 µl of all reactants were added to duplicate wells of 96-well microplates (Nunc MaxiSorp®, Nalge Nunc International, Rochester, NY). Serial dilutions of Vaccinia virus in PBS with 0.05% glutaraldehyde were coated on wells by 18 h incubation at 4 °C. All further incubations were performed at 24 °C. Plates were washed one time with PBS with 0.1% Tween 20 (PBST) and blocked using blocking buffer (2 mg/ml casein, 2 mg/ml bovine serum albumin in PBS). Plates were washed again, then serial dilutions of rabbit anti-Vaccinia antibody were added and incubated for 30 min. Wells were washed three times with PBST, and horseradish peroxidase (HRP) labeled anti-rabbit antibodies in either a 1:500 or 1:2000 dilution were added and incubated for 30 min. Wells were washed three times with PBST, and QuantaBlue substrate (Pierce Biotechnology) was added and incubated for 20–25 min. QuantaBlue stop solution was then added, and fluorescence, detected as relative fluorescence units (RFU), was measured at 325 nm excitation and 420 nm emission using a Spectra Max Gemini XS fluorometer (Molecular Devices, Sunnyvale, CA). Fluorescent values for all blanks (reactants minus antigen) were subtracted from all corresponding samples to

determine the signal over background. Any value greater than two standard deviations plus the average background was considered a positive signal.

#### 2.4. Sample preparation of throat swab specimens

Throat swab specimens were taken by swabbing the rear pharyngeal walls of volunteers with a sterile rayon swab. One throat swab specimen from each volunteer was used as a negative control. The negative control swab was immersed in 1 ml of PBS in a microfuge tube. The swab was vigorously swirled in the PBS, then carefully squeezed against the side of the tube to remove as much liquid as possible from the swab. This suspension was run through the biosensor as a negative control, prior to running the corresponding seeded samples from each volunteer.

Seeded throat swab specimens were prepared by two different methods. The first method of dilution entailed immersing one swab into 50  $\mu$ l PBS containing  $2.5 \times 10^8$  pfu Vaccinia virus, bringing this suspension to 1 ml with PBS and making serial dilutions. In the second method, a separate throat swab specimen from each volunteer was immersed into decreasing Vaccinia virus concentrations. One swab was dipped into a volume of 50  $\mu$ l containing  $2.5 \times 10^6$  pfu Vaccinia virus. A second swab was dipped into a volume of 50  $\mu$ l containing  $2.5 \times 10^5$  pfu Vaccinia virus. The third swab was dipped into a volume of 50  $\mu$ l containing  $2.5 \times 10^4$  pfu Vaccinia virus. All swabs were allowed to absorb all of the solution. Each swab was then carefully squeezed against the inside of another tube containing 950  $\mu$ l of PBS. All swabs were swirled vigorously and then squeezed against the inside of their respective tubes removing as much liquid as possible. Each tube was brought to 1 ml total volume with PBS.

#### 2.5. Instrumentation

The Analyte 2000 is a microprocessor controlled, four cuvette, single wavelength fluorometer designed for evanescent wave fluoroimmunoassays. A 635 nm laser diode pulses the excitation light through a single fiber exciting fluorescent molecules within 100–1000 nm of the waveguide surface. Emission light is coupled back through an excitation fiber through a long pass filter, a focusing lens, a dichroic filter, and a second focusing lens onto a photodiode. The two filters eliminate any recoupled excitation light. The electrical signal from the photodiode is conducted to the analyte signal processing electronics and expressed as picoAmperes (pA).

#### 2.6. Polystyrene waveguide preparation

Polystyrene waveguides, each 4 cm in length, were obtained from Research International and prepared as follows: the waveguides were cleaned by sonication with a solution of isopropanol for 30 s, rinsed with deionized water and air dried. The distal ends of the waveguides were dipped in flat

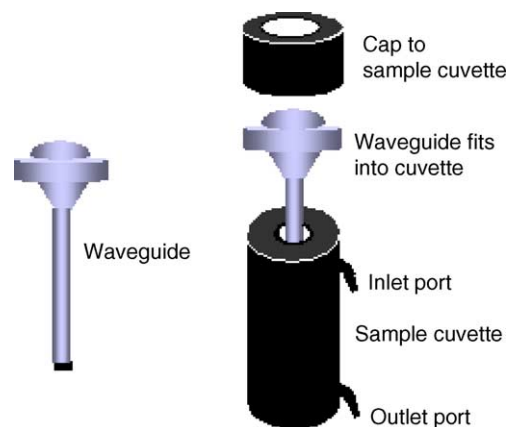


Fig. 1. The waveguide is mounted inside a sample cuvette that firmly locks into an adapter.

black paint and allowed to dry. When the waveguides were completely dry, they were placed into reaction chambers made from 100  $\mu$ l glass capillary tubes cut to a length able to contain the waveguide. Streptavidin solution (100  $\mu$ g/ml) prepared in PBS was used to fill the 100  $\mu$ l volume contained in the reaction chambers. Chambers containing the streptavidin solution and waveguides were incubated at 4  $^{\circ}$ C for 18–72 h.

#### 2.7. Vaccinia virus in PBS biosensor assays

Streptavidin-coated polystyrene waveguides were placed into sample cuvettes (Fig. 1). The cuvettes were then clamped into the adapter portion of the Analyte 2000. One milliliter of sterile PBST was injected with a syringe into the sample cuvette. This rinse removed non-adsorbed streptavidin from the waveguide. Two hundred microliters of 200  $\mu$ g/ml biotin labeled anti-Vaccinia antibody was injected into the cuvette and incubated at 24  $^{\circ}$ C for 30 min. An additional 200  $\mu$ l of capture antibody was injected and incubated for 30 min. Following the second incubation, the waveguide was rinsed with 1 ml PBST to remove any unbound antibody. To determine the background signal, detection antibody (10  $\mu$ g/ml Cy5-labeled anti-Vaccinia antibody) in blocking buffer (PBS containing 2 mg/ml bovine serum albumin, 2 mg/ml casein) was injected, incubated for 5 min, rinsed two times and readings in picoAmperes were taken. These incubations were repeated four times to obtain four background readings. One milliliter of the Vaccinia virus sample was incubated for 10 min at 25  $^{\circ}$ C. The waveguide was rinsed once with 1 ml PBST. Detection antibody was then added, incubated for 5 min and rinsed two times. A reading was taken following the final rinse. Background signals were subtracted from this reading to obtain the final change in signal above background (in pA). Assays were always performed on each waveguide with the sample having the lowest viral concentration assayed first and the sample having the highest viral concentration assayed last. Twenty assays (sets of four) were performed in this manner.

## 2.8. Biosensor assay data analysis

The limit of detection was designated as three times the standard deviation of the last three background signals. A change in signal above background for all samples tested was considered a positive result if the change was higher than the limit of detection. To normalize the signals, the change in signal above background for each sample was divided by the change in signal above background of the highest virus concentration ( $2.5 \times 10^7$  pfu/ml) tested. The change in signal above background for the  $2.5 \times 10^7$  pfu/ml Vaccinia virus sample was arbitrarily set to 100. The following calculation was used: change in signal above background for sample tested/change in signal above background for sample containing  $2.5 \times 10^7$  pfu/ml Vaccinia virus  $\times 100$ . The mean and standard deviation for the normalized change in signal above background for four waveguides was calculated for each viral concentration tested. Each assay was performed at least two times.

## 3. Results

### 3.1. ELISA assays

Three separate ELISAs were carried out using Vaccinia virus as the target antigen. Antigen concentrations ranged from  $1.3 \times 10^1$  to  $1.3 \times 10^8$  pfu/well in all assays. Any value greater than two standard deviations plus the average background was considered a positive signal. Detection occurred at  $1.3 \times 10^4$  pfu/well (Fig. 2).

### 3.2. Vaccinia virus in PBS biosensor assays

The sensitivity for detection of Vaccinia virus diluted in PBS using the biosensor was determined. Assay results are shown in Table 1. Table 1 shows the normalized mean

Table 1

Results of a typical biosensor assay (four channels) for the detection of Vaccinia virus in seeded PBS buffer

Vaccinia concentration (pfu/ml)	Mean change in signal above background (pA) <sup>a</sup>	Mean normalized signal <sup>b</sup>
PBS only (negative)	$-13.2 \pm 7.1^c$	$63.3 \pm 3.14$
$2.5 \times 10^2$	$-8.7 \pm 6.3$	$63.8 \pm 4.3$
$2.5 \times 10^3$	$-7.9 \pm 9.9$	$64.1 \pm 3.4$
$2.5 \times 10^4$	$11.0 \pm 16.4$	$67.0 \pm 2.7$
$2.5 \times 10^5$	$54.3 \pm 18.8$	$73.8 \pm 1.4$
$2.5 \times 10^6$	$122.2 \pm 26.2$	$84.3 \pm 1.0$
$2.5 \times 10^7$	$223.4 \pm 24.6$	100.0

<sup>a</sup> Limit of detection = 24.5.

<sup>b</sup> Limit of detection = 72.9.

<sup>c</sup> One standard deviation.

from four channels (cuvettes) of a representative biosensor assay for the detection of Vaccinia virus in PBS buffer. Vaccinia virus detection occurred at  $2.5 \times 10^5$  pfu/ml or greater in 14 out of 14 assays. Detection was positive in 5 out of 14 assays or 35% of the time at concentrations of  $2.5 \times 10^4$  pfu/ml.

### 3.3. Biosensor throat swab assays

Vaccinia virus-seeded throat swab specimens were tested because infection normally occurs after primary implantation of the virus on the oropharyngeal or respiratory mucosa. Vaccinia virus-seeded PBS mixed with oropharyngeal exudate was assayed using the biosensor. The mean results of four biosensor assays (16 channels) for detection of the Vaccinia virus on seeded throat culture swabs is shown in Table 2.

Detection at levels of  $2.5 \times 10^5$  pfu/ml and greater were consistent and reproducible for Vaccinia virus detection when virus was suspended in PBS or in throat swab solutions.

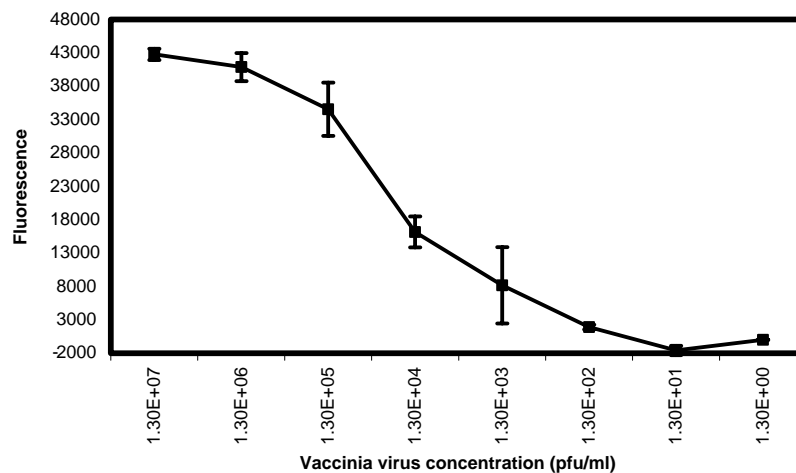


Fig. 2. ELISA showing relative fluorescence with decreasing Vaccinia virus concentration. The Vaccinia virus antibody is a rabbit polyclonal antibody. The secondary HRP labeled antibody is an anti-rabbit IgG.

Table 2

Results of four biosensor assays for the detection of Vaccinia virus in seeded throat culture swabs

Vaccinia concentration (pfu/ml)	Mean change in signal above background (pA) <sup>a</sup>	Mean normalized signal <sup>b</sup>
PBS only (negative)	$-18.1 \pm 18.7^c$	$78.2 \pm 12.2$
$2.5 \times 10^4$	$1.6 \pm 11.9$	$80.9 \pm 11.1$
$2.5 \times 10^5$	$35.4 \pm 44.1$	$84.1 \pm 9.3$
$2.5 \times 10^6$	$89.1 \pm 96.8$	$88.3 \pm 5.8$
$2.5 \times 10^7$	$193.8 \pm 180.3$	100.0

<sup>a</sup> Limit of detection = 17.4.

<sup>b</sup> Limit of detection = 84.0.

<sup>c</sup> One standard deviation.

#### 4. Discussion

Enzyme-linked immunosorbent assays (ELISAs) can be used to detect Vaccinia virus. However, the total minimum detection time using ELISA is 24 h because an initial 18 h incubation step is required to bind virus to wells. A recently developed real-time PCR assay for the detection of smallpox virus DNA (Espy et al., 2002) can detect 5–10 copies of target DNA in a 20  $\mu$ l sample. It is unclear whether this result would directly correlate to 250–500 organisms/ml. Real time PCR can provide a definitive answer within 2 h of receipt in the laboratory. However, PCR requires trained personnel, clean samples, isolated conditions, and the specimen must reach the laboratory in a short amount of time.

The total time required for the detection of Vaccinia virus in a single sample using the biosensor assay was 20 min. Preparation of waveguides and background readings were performed in advance of the actual assay. Specimen preparation prior to testing the sample was minimal and involved removal of virus particles from throat swabs by stirring each swab in a buffer solution. The throat swab samples could then be run directly with the biosensor. Infected persons shed most smallpox virus particles from the epithelium of the naso-oropharynx and salivary glands (Dixon, 1962; Suvakovic and Kecmanovic, 1976; Henderson et al., 1999; Smee et al., 2001; Whitby et al., 2002; Baron, 2003). The titer of infectious viral particles shed from a patient's naso-oropharynx ranges from  $10^6$  to  $10^8$  pfu/ml. If we consider that one or more viral particles form a plaque, this titer is 10–100 times higher than the detection limit of the fiber optic biosensor assay described in this report. In this study, the lower limit of Vaccinia virus detection from throat swab specimens for the biosensor was  $2.5 \times 10^5$  pfu/ml. Therefore, the biosensor assay should be capable of detecting the virus from infected patient's throat swab specimens.

An advantage to using the biosensor assay described in this report is that the assays can be performed on site using the RAPTOR (Research International), an automated sister model of the Analyte 2000. The RAPTOR is portable, self-contained, and requires minimal training (Golden et al., 1997; Lim, 2003). Accuracy and speed are both vital in

the diagnosis of smallpox (Madeley, 2003). A false positive could produce widespread panic whereas a delayed diagnosis could be fatal to the patient and could delay quarantine of infected individuals resulting in a pandemic. A sensitive, rapid uncomplicated method of biodetection is needed.

#### 5. Conclusion

If a bioterrorism event were ever suspected, detection of the smallpox virus from the nasopharyngeal wall would allow a diagnosis before manifestation of the disease. The titer of infectious viral particles shed from a patient's naso-oropharynx ranges from  $10^6$  to  $10^8$  pfu/ml. If we consider that one or more viral particles form a plaque, this titer is 10–100 times higher than the detection limit of the fiber optic biosensor assay described in this report. In this study, the lower limit of Vaccinia virus detection from throat swab specimens for the biosensor was  $2.5 \times 10^5$  pfu/ml. Therefore, the biosensor assay should be capable of detecting the virus from infected patient's throat swab specimens. Vaccination could then be administered to patients whose throat swab samples exhibited positive detection. Vaccination would begin within 4 days of exposure and would provide some protection from the disease state and significant protection from a fatal outcome (Dixon, 1962; Mortimer, 2003), and may possibly prevent further spread of the virus. This work shows that an evanescent wave biosensor has the potential to fulfill the definite need for rapid on-site detection capabilities for smallpox virus.

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