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Rapid Identification of Swine-Origin Influenza A Virus by Peptide Nucleic Acid Chromatography

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Abstract

Early and rapid detection of a virus infection is crucial for preventing transmission and initiating treatment. The sequence-specific detection of a target viral gene has been used to diagnose not only a virus infection, but also the virus strain, clade, and drug resistance. We here propose a novel type of rapid diagnostic that can visually identify virus infections using nucleic acid hybridization-based methods.

Keywords: Diagnosis; Virus infection; Viral gene; Polymerase chain reaction; Microarray; Nucleic acid; Hybridization; Enzyme-linked immunosorbent assay

Abbreviations: BCIP: 5-Bromo-4-Chloro-3'-Indolylphosphatase p-toluidine salt; IgG: Immunoglobulin G; LAMP: Loop-mediated isothermal amplification; NBT: Nitro-Blue Tetrazolium chloride; NP: Nucleoprotein; NS: Nonstructural protein; Pfu: Plaque formation unit; PNA: Peptide Nucleic Acid

Introduction

Before the development of molecular biology techniques, the laboratory diagnosis of virus infections required the isolation of the virus in a cell culture system. Methods for the detection of viruses by cell-based virology tests were based on the cytopathic effect, neutralization, hemadsorption or hemagglutination. Such cell-based assays can only be performed in biosafety facilities with established cell culture systems.

The development in the late 1980s of the Polymerase Chain Reaction (PCR) allowed the rapid identification of virus pathogens together with information on the virus gene sequence [1]. Information on the virus gene is useful for not only identifying the origin of the virus pathogen from an epidemiological viewpoint, but also for predicting the efficacy of antiviral agents. Since PCR-based assays are sensitive compared to other diagnostic methods, PCR has become the gold standard for the laboratory-based diagnosis of virus infections.

TaqMan® probe is a more sequence-specific method than other PCR-based methods because the probe induces fluorescence only as the 5′-end fluorophore is cleaved by the exonuclease activity of Taq DNA polymerase [2]. However, this method cannot be applied to multiplex assays.

Loop-Mediated Isothermal Amplification (LAMP) expanded the nucleic acid-based diagnosis of virus infections. This technique amplifies the target virus gene without the need for a thermal cycler; furthermore, viral gene amplification can be monitored by both fluorescence emission from a DNA intercalator and the increased turbidity of magnesium pyrophosphate in the reaction tube [3]. The challenge in using this technique, however, is the design of four primers complementary to the virus gene conserved sequences. These primers are required for the strand exchange amplification reaction.

DNA microarrays are a powerful tool for identifying virus infections because the sequence specificity is confirmed at both the RT-PCR and annealing steps [4]. However, the sensitivity of the technique for identifying viruses relies on the efficiency of target amplification and hybridization of the amplicons and probes. Furthermore, 1-2 days are required for the assay.

Recently, Zhao et al. [5] reported a method to detect a virus gene without using PCR or DNA elongation reactions. They developed a gold nanoparticle-based genomic microarray assay and identified swine-origin influenza A virus (H1N1/2009) using three different oligonucleotides [ref.]. Specific hybridization between a viral RNA, a capture oligo and an intermediate oligo results in the formation of a sandwich complex. The poly-A region is then labeled with the gold nanoparticle probed poly-T, followed by staining with a silver enhancer. The light-scattering signal from the silver-enhanced gold nanoparticle is captured by a photosensor. Using this assay, Zhao et al. could detect 0.5 μg of purified target viral RNA without using any enzymatic reaction.

All these methods require specialized equipment and therefore are not practical for point-of-care diagnosis. Currently, there is no method for capturing intact influenza viral genome RNA on a device and providing a signal visible with the naked eye. We therefore propose a novel type of diagnostic that can rapidly and visually identify a virus gene by nucleic acid hybridization-based methods.

Visual and Direct Identification of Virus Genome RNA by PNA

Peptide nucleic acid (PNA) is a DNA/RNA mimic in which the phosphate backbone has been replaced by a neutral amide backbone composed of N-(2-aminoethyl)glycine linkages [6]. The advantages of PNAs for molecular recognition are their high binding affinity [6], good mismatch discrimination [7], nuclease and protease resistance [8], and low affinity for proteins [9]. These chemical properties are useful for detecting a virus gene in a clinical specimen. Furthermore, hairpin-type PNA composed of two homopyrimidine PNA strands connected

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via a linker molecule can form a stable triplex with a complementary homopurine strand via Watson-Crick and Hoogsteen base pairing [10].

As shown in Figure 1a, we designed a hairpin-type PNA targeting a highly conserved sequence of the nonstructural protein (NS) gene of swine-origin influenza A virus (H1N1/2009). The hairpin-type PNA recognizes the NS gene within the virus lysate in a sequence-specific manner [11]. The NS gene forms ribonucleoprotein complexes with nucleoprotein (NP) [12]. The NS gene is captured by the hairpin-type PNA immobilized on a well and is detected using two antibodies: a monoclonal anti-NP IgG primary antibody which has cross-reactivity with a broad spectrum of influenza A/H1N1 viruses, and a polyclonal goat anti-mouse IgG secondary antibody conjugated with alkaline phosphatase (Figure 1a). The phosphatase produces a purple dye in the presence of BCIP/NBT assay solution. Using the colour change observed with this ELISA-assisted PNA-array, we succeeded in visually identifying swine-origin influenza A virus (H1N1/2009) from several other seasonal viruses (A/H3N2, B) [11]. Sequence specificity is guaranteed by use of the PNA, and the sensitivity is increased by the ELISA-assisted system. The virus detection limit is 10^4 pfu, making this approach useful for the identification of viruses in clinical specimens. However, 2-3 h is required for the virus incubation, antibody incubation, and washing procedures.

To address these issues, we developed a novel diagnostic that can visually subtype influenza A virus by a hairpin-type PNA-immobilized chromatography system using a gold-conjugated anti-NP antibody (Figure 1b). Our gold-conjugated antibody-assisted PNA chromatography methodology detected the virus gene at a titer of 6×10^4 and 1×10^4 pfu on the test line within 5 min, without the need for wash or incubation steps (Figure 1c). The band intensities of the test line indicated that the virus gene detection limit is approximately 1×10^4 pfu, which is 10-fold less sensitive than commercially available immunochromatography test kits for influenza A virus. Indeed, our PNA chromatography methodology contains an immunochromatography system on the control line: the viral nucleoprotein (NP) was directly captured by the anti-NP antibody immobilized on the control line, and then the captured NP was visualized by the gold-conjugated anti-NP antibody in the same manner as conventional immunochromatography test kits. To estimate the virus gene detection efficiency of the PNA chromatography step, we compared the band intensities of the control and test lines using virus titers of 6×10^4 and 1×10^4 pfu and found that the band intensities of the control lines were identical and of maximum intensity under these conditions (Figure 1c). On the other hand, the band intensities of the test lines were different depending on the virus titer. When the virus titer was 1×10^4 pfu, the intensity of the test band was approximately 10-fold lower compared to the control line, whereas when the virus titer was 6×10^4 pfu, the intensity of the test line was approximately 5-fold lower than the control line. These results indicated that although our PNA chromatography is somewhat less sensitive than conventional immunochromatography, it can still

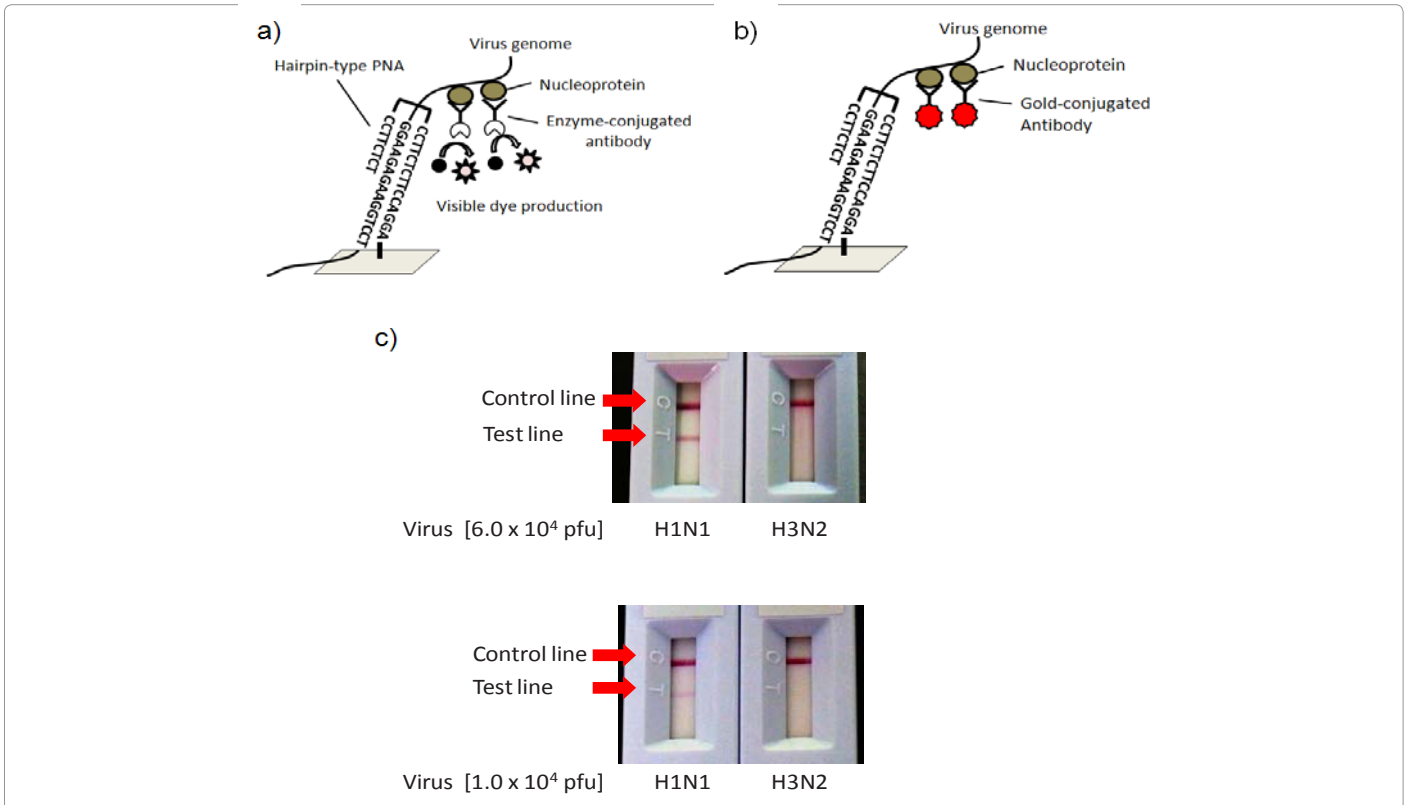

Figure 1: Schematic overview of two different PCR-free virus gene detection methods based on nucleic acid-based hybridization technologies.

a) ELISA-assisted PNA array. The target virus genome is captured by a hairpin-type PNA and the accompanying viral nucleoprotein is labeled with anti-NP antibodies and conjugated alkaline phosphatase. Purple dye production by the phosphatase can be detected in the BCIP/NBT assay solution added to the well. b) Gold-nanoparticle-assisted PNA chromatography. The target virus genome is captured by hairpin-type PNA and the NP protein is labeled with anti-NP antibody modified with a gold-nanoparticle. c) Virus genome-specific identification of swine-origin influenza A/H1N1 virus using gold-nanoparticle assisted PNA chromatography. The control line detects the NP protein of the H1N1 and H3N2 strains of influenza A virus, while the test line detects the viral RNA/NP complex of swine-origin influenza A virus (H1N1/2009) at a virus titer of 6.0×10^4 pfu and 1.0×10^4 pfu.

Method	Description
ELISA-assisted PNA array	Advantages -No viral RNA purification necessary -Sensitive (detection limit:10^4 pfu[a]) -Detect viral gene presence by the naked eye Disadvantages -ELISA system anti-NP antibody is necessary -Diagnosis requires 2-3 hours.
Gold-conjugated antibody-assisted PNA chromatography	Advantages -Rapid (result can be obtained in 5 min) -No virus purification, incubation or washing necessary -Sensitive (detection limit: 10^4 pfu) -Detect viral gene presence by the naked eyes Disadvantages -Gold-nanoparticle conjugated anti-NP antibody is necessary

[a]Plaque forming unit

Table 1: Diagnosis of swine-origin influenza A virus (H1N1/2009) by PCR-free viral RNA detection methods.

detect the virus gene from clinical specimens obtained from influenza A virus-infected patients.

The advantages and disadvantages of the gold nanoparticle-assisted DNA microarray and ELISA-assisted PNA methods are summarized in Table 1. Both methods hold promise for point-of-care application because neither requires PCR amplification or DNA elongation reactions.

Although these assays are applicable only to detecting influenza viruses, further modification of these method will enable us to provide rapid, simple, sensitive and selective diagnostic tools for a broad spectrum of virus pathogens.

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