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Research paper

Immuno-PCR for one step detection of H5N1 avian influenza virus and Newcastle disease virus using magnetic gold particles as carriers

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ABSTRACT

Detecting avian influenza virus (AIV) and Newcastle disease virus (NDV) at low concentrations from tracheal and cloacal swabs of avian influenza- and Newcastle disease-infected poultry was carried out using a highly sensitive immunological-polymerase chain reaction (immuno-PCR) method. Magnetic gold particles were pre-coated with a capture antibody, either a monoclonal anti-AIV/H5 or monoclonal anti-NDV/F and viruses serially diluted ten-fold from 10^2 to 10^{-5} EID_{50}/ml. A biotinylated detection antibody bound to the viral antigen was then linked *via* a streptavidin bridge to biotinylated reporter DNA. After extensive washing, reporter DNA was released by denaturation, transferred to PCR tubes, amplified, electrophoresed and visualized. An optimized immuno-PCR method was able to detect as little as 10^{-4} EID_{50}/ml AIV and NDV. To further evaluate the specificity and the clinical application of this IPCR assay for AIV H5N1 and NDV, the tracheal swab specimens, taken from chickens which were infected with H5N1/AIV, H9N2/AIV, H7N2/AIV, NDV, IBDV, IBV/H_{120}, were detected by IPCR. Our data demonstrated that this monoclonal antibody-based immuno-PCR method provides a platform capable of rapid screening of clinical samples for trace levels of AIV H5 and NDV in one step.

1. Introduction

Highly pathogenic avian influenza (HPAI) and Newcastle disease (ND) are two extremely contagious, multi-organ systemic diseases of poultry with high mortality. They are caused by H5 and H7 subtypes of type A influenza virus (AIV) and Newcastle disease virus (NDV), respectively. AIV and NDV have emerged in poultry and wildlife worldwide, causing sporadic but serious and devastating outbreaks. Moreover, HPAI and ND tend to present simultaneously causing some difficulties in diagnosing these diseases. The accurate and prompt diagnosis of AIV (H5N1) and NDV infection in birds is the critical component of a disease control plan.

The diagnosis of HPAI and ND are traditionally based upon the isolation and characterization of the virus. Virus isolation in embryonated eggs is a conventional method that tends to be costly, labor intensive, and require special collection and transport conditions to ensure virus viability. Although there have been enormous developments and improvements in molecular and

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other diagnostic techniques, such as reverse transcription-polymerase chain reaction (RT-PCR) or quantitative RT-PCR (qRT-PCR) (Creelan et al., 2002; Das et al., 2006; Farkas et al., 2009; Gohm et al., 2000; Hoffmann et al., 2009; Wise et al., 2004; Xiang et al., 2007), these methods are technically demanding, and false-positive results may arise from cross contamination between samples. Consequently, they are not suited for wide application, especially for those laboratories without trained personnel and at biosafety level 3. To improve the ability to detect AIV and NDV, laboratories need more rapid and less cumbersome methods for the direct identification of AIV and NDV in clinical specimens.

Fortunately, a very sensitive antigen detection system, termed immuno-polymerase chain reaction (immuno-PCR, IPCR) was developed by Sano et al. (1992). The immuno-PCR is a method that combines the specificity of immunological detection methods with the exponential amplification of PCR. Since then, immuno-PCR use has expanded and has been successfully used to detect oncogenes (Zhou et al., 1993), T cell receptors (Sperl et al., 1995), angiotensinogen (Sugawara et al., 2000), tumor necrosis factor α (Komatsu et al., 2001; Saito et al., 1999), pathogenic *Escherichia coli* (Ogunjimi and Choudary, 1999), and equine influenza A viruses (Ozaki et al., 2001), viral antigens (Maia et al., 1995; Mweene et al., 1996), *Clostridium botulinum* neurotoxin (Wu et al., 2001), Shiga toxin (Zhang et al., 2008), and parasites (Chye et al., 2004). This method is similar to the enzyme-linked immunosorbent assay (ELISA), however, instead of using an enzyme that is conjugated to an antibody, a reporter DNA molecule is used in immuno-PCR. Since the reporter DNA can be amplified by PCR, sensitivity can be increased 10^2–10^5-fold (Joerger et al., 1995; Sano et al., 1992). At present, IPCR is regarded as one of the most sensitive detection methods for trace amounts of protein.

In this study, an immuno-PCR method was designed for the detection of extremely low concentrations AIV subtype H5, and NDV. All procedures were performed in a biosafety level 3 facility.

2. Materials and methods

2.1. Preparation of AIV (H5 subtype) and NDV

AIV (H5 subtype) and NDV were inoculated into the allantoic cavity of 9–11-day-old specific pathogen-free (SPF) embryonated fowl eggs and incubated at 37 °C for 4 days. The allantoic fluid of any eggs containing dead or dying embryos during the incubation and all eggs at the end of the incubation period were collected. The titers of AIV and NDV were determined through 50% egg infective dose (EID_{50}) to be approximately 10^3 EID_{50}/ml and 10^4 EID_{50}/ml, respectively. NDV and AIV were serially diluted ten-fold in PBS ranging from 10^2 to 10^{-5} EID_{50}/ml.

2.2. Preparation and purification of biotinylated reporter DNA

Two biotinylated double-stranded reporter DNAs were generated and designated AIV-Reporter DNA and NDV-Reporter DNA by PCR amplification of DNA-218 and DNA-328 with a forward primer (5′-biotin-GGG ATA ACG CAG GAA AGA A-3′) and a non-biotinylated reverse primer (5′-CAG GGT CGG AAC AGG AGA-3′). PCR was performed according to the manufacturer's instructions (Takara one shot LA PCR™ mix). Each 50 μl PCR contained 10 mM Tris-HCl buffer (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.4 mM each dNTP, 25 pM of each primer, 2.5 U Taq DNA polymerase and 25 ng template DNA (DNA-218 or DNA-328). The thermal cycling involved 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min. The PCR products were purified with a DNA Fragment Purification kit (Takara).

The sequences of DNA-218 and DNA-328 were as follows: 5′-GGG ATA ACG CAG GAA AGA ACA TGT GAG CAA AAG GCC AGC AAA AGG CCA GGA ACC GTA AAA AGG CCG CGT TGC TGG CGT TTT TCC ATA GGC TCC GCC CCC CTG ACG AGC ATC ACA AAA ATC GAC GCT CAA GTC AGA GGT GGC GAA ACC CGA CAG GAC TAT AAA GAT ACC AGG CGT TTC CCC CTG GAA GCT CCC TCG TGC GCT CTC CTG TTC CGA CCC TG-3′ (DNA-218); and 5′-GGG ATA ACG CAG GAA AGA ACA TGT GAG CAA AAG GCC AGC AAA AGG CCA GGA ACC GTA AAA AGG CCG CGT TGC TGG CGT TTT TCC ATA GGG TGG TTA CGC GCA GCG TGA CCG CTA CAC TTG CCA GCG CCC TAG CGC CCG CTC CTT TCG CTT TCT TCC CTT CCT TTC TCG CCA CGT TCG CCG GCT TTC CCC GTC AAG CTC GCT CCG CCC CCC TGA CGA GCA TCA CAA AAA TCG ACG CTC AAG TCA GAG GTG GCG AAA CCC GAC AGG ACT ATA AAG ATA CCA GGC GTT TCC CC CTG GAA GCT CCC TCG TGC GCT CTC CTG TTC CGA CCC TG-3′ (DNA-328).

2.3. The coupling of capture antibody with magnetic gold particles

Capture antibodies, Mouse monoclonal anti-AIV/H5 IgG and anti-NDV/F IgG, were respectively coated onto gold magnetic particles according to the manufacturer's instructions (GoldMag™-AS). The coating procedure involved diluting the capture antibody in coupling buffer (provided by the manufacturer) to 1 μg/μl in a 400 μl volume. An 80 μl volume of this dilution was transferred to a microcentrifuge tube and marked as '***pre***'. A 300 μl volume of diluted antibody was added to the magnetic particle solution and incubated at 180 rpm and 37 °C for 20 min. After magnetic isolation, the supernatant was collected and marked as '***post***'. Magnetic particles coupled to the desired antibody were washed twice with washing buffer provided by the manufacturer. The two wash solutions were collected and marked as '$\boldsymbol{wash_1}$' and '$\boldsymbol{wash_2}$', respectively. Non-adsorbed sites on the surface of magnetic beads were blocked with 300 μl blocking buffer, provided by the manufacturer, containing 4.5% skim milk powder, 5 mM EDTA and 1 mg/ml herring sperm DNA for 30 min at 37 °C with shaking. After magnetic beads were washed five times, preservation buffer (provided by the manufacturer) was added and samples stored at 4 °C. The absorbance of the '***pre***' and '***post***' were determined at 280 nm with the coupling buffer as a blank control, and the absorbance of '$\boldsymbol{wash_1}$' and '$\boldsymbol{wash_2}$' were determined at 280 nm with washing buffer as a blank control. According to the following formula, the coupling