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Development of latex agglutination test with nucleoprotein as antigen for detection of antibodies to swine influenza virus ☆

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ABSTRACT

As pigs are susceptible to infection with both avian and human influenza A viruses, they have been proposed to be an intermediate host for the generation of pandemic virus through reassortment. The broad susceptibility of pigs to influenza viruses emphasizes the importance of surveillance of swine influenza virus. Thus, A latex agglutination test (LAT) was developed for rapid detection of antibodies to swine influenza virus. The nucleoprotein (NP) gene of the H9N2 swine influenza virus isolated from local farms was cloned, and expressed in *Escherichia coli*. Reactivity of the expressed protein was confirmed by Western blot. Subsequently, the NP gene was purified and used as the diagnostic antigen to develop a NP-based LAT for detecting antibodies to swine influenza virus. The LAT is shown to be specific for swine influenza virus and does not cross-react with swine sera that have antibodies to other swine viruses. The NP-LAT and HI test had a high agreement ratio in detecting 10 serum samples from naïve pigs, 28 serum samples from experimentally infected and vaccinated pigs. Compared with the hemagglutination inhibition (HI) test, the corresponding specificity, sensitivity, and correlation were 92.9%, 94.1%, and 94.1%, respectively, in detecting 321 serum samples from vaccinated pigs. The NP-LAT developed in our laboratory is a rapid and simple test suitable for field monitoring of antibodies to swine influenza virus. We conclude that it was specific and sensitive and it has great application potential in China's long-term prevention and control of swine influenza virus.

1. Introduction

Swine influenza is an acute respiratory disease caused by influenza A virus within the Orthomyxoviridae family. Infection of pigs with influenza A viruses is of substantial importance to the swine industry and to the epidemiology of human influenza [1]. The influenza A viruses are classified further into subtypes on the basis of the antigenicities of their surface glycoprotein hemagglutinin (HA) and neuraminidase (NA). To date, 16 HA (H1–16) and 9 NA (N1–9) subtypes of these viruses have been identified [2]. At present, the H9N2 subtype virus is a notable member of the influenza family because it can infect not only chickens, ducks and pigs, but also humans [3–9]. In China, the H9N2 virus was first isolated from a chicken in Guangdong province in 1992 and now is the most prevalent subtype of influenza virus in poultry in China [4]. Recent studies have demonstrated that H9N2 virus can infect pigs and cause significant morbidity and mortality [4,8]. In southern China, at least 2% of blood donors tested were positive for H9 antibodies [3,10], suggesting that human infection with H9N2 occurs ubiquitously in this area. The frequent occurrence of influenza outbreaks among humans, swine, and birds has not only caused a huge economic loss but also posed a severe threat to human health. The broad susceptibility of pigs to influenza viruses emphasizes the importance of surveillance of swine influenza virus. Thus, a diagnostic assay is needed to efficiently diagnose swine influenza virus infection and at the same time, to detect antibodies induced by vaccines to validate vaccine efficacy. Currently, the serologic tests for swine influenza virus antibodies recommended by the World Organisation for Animal Health (OIE) are hemagglutination inhibition (HI) and an agar gel precipitation test (AGPT). This procedure, however, is labor intensive and time consuming. Moreover, hemagglutinin of swine influenza virus is subtype specific and also is undergoing constant mutation; HI assay is limited in its ability to detect all swine influenza virus subtypes. In serological monitoring, detection of antibodies against the nucleoprotein (NP) and the matrix protein (M1), which are highly conserved among influenza A viruses, should be attempted first, because this procedure can detect infections with various swine influenza virus subtypes. The AGPT is used widely because of its simplicity and broad specificity for the detection of influenza A viruses. However, with the AGPT, the final results are obtained after at least 2 days. Another serological test used commonly for influenza is the enzyme-linked immunosorbent assay (ELISA), which measures antibody responses to conserved proteins. ELISA results are obtained after

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several hours, and the ELISA procedure requires the use of special instruments and may be relatively labor intensive. In addition, ELISA may also show nonspecific reactions in some cases.

Recently, a latex agglutination test (LAT) for detection of antibodies against the H5 subtype viruses was reported [11,12]. However, there are no reports on rapid and simple diagnostic methods for detecting antibodies against various subtypes of swine influenza virus. In this study, a NP based type-specific LAT was developed for detecting antibodies against all subtype influenza A viruses in our laboratory, and it was used in the diagnosis and serologic epidemiological investigations of swine influenza virus. The assay is sensitive, specific, and relatively inexpensive. It has great potential to be widely used by the pig industry of Asia.

2. Materials and methods

2.1. Virus

The A/swine/HeBei/012/2008/(H9N2) virus was isolated by our laboratory from local farms in Hebei province of China in 2008. Viruses were subtyped by standard hemagglutination-inhibition and neuraminidase-inhibition tests. The viruses were passaged in embryonated chicken eggs and the allantoic fluids were harvested and used as stock viruses for further analysis.

2.2. Experimental sera

H1N1, H3N2, and H9N2 positive sera were obtained from the Harbin Veterinary Research Institute. Positive sera for porcine reproductive and respiratory syndrome virus (PRRSV) and classic swine fever virus (CSFV) were obtained from the Harbin Veterinary Research Institute, positive sera for porcine pseudorabies virus (PRV) were obtained from the Lab of Animal Infectious Diseases, Huazhong Agricultural University.

14 pigs were experimentally infected by the oronasal route with $10^6 TCID_{50}$ of MDCK cell culture-grown H9N2 influenza viruses in 1 ml cell culture medium using a nebulizer device (Wolfe Tory Medical) as previously described [13]. The study was approved by the Animal Care and Use Committee of the Hebei North University (Zhangjiakou, Hebei). All animal procedures followed the ethics guidelines of the National Research Council Guide for the Care and Use of Laboratory Animals (1985). Prior to infection animals were tested seronegative for influenza antibodies in a commercial blocking ELISA (ID.Vet). Blood samples used in further serological studies were obtained on day 28 post inoculation (p.i.).

10 sera samples from naïve pigs (unvaccinated and showed no clinical episodes of infection;), and 14 anti-H9N2-positive sera (HI titer $>2^4$) from experimentally vaccinated pigs were prepared by our laboratory. Blood samples used in further serological studies were obtained on the 28th day.

2.3. Field sera

A total of 321 porcine field sera samples were collected from vaccinated pigs in several field farms in China.

2.4. NP gene cloning and nucleotide sequence analysis

Viral RNA was extracted from allantoic fluid using the RNA-SOLV® reagent RNA isolation solvent (Omega Bio-tek, Lilburn, GA) according to the manufacturer's instructions. For the amplification of the NP gene, we designed a primer pair: forward primer, 5′-GCG GAT CCG CGT CTC AAG GCA CCA AAC-3′ (containing the BamH I cleavage site) and reverse primer, 5′-CGC AAG CTT ATA GTC ATA CTC CTC TGC-3′ (containing the Hind III cleavage site). Then the complete open reading frame of the NP gene was amplified using reverse transcriptase-polymerase chain reaction (RT-PCR). The amplified DNA fragments were purified and sequenced on an ABI Prism 377XL DNA sequencer. The sequence homology was analyzed using the Basic Local Alignment Search Tool (BLAST) program of GenBank from the National Center of Biotechnology Information.

2.5. NP protein expression and purification

A recombinant expression plasmid which is named pGEX-KG/NP was obtained by inserting NP gene into a prokaryotic expression plasmid pGEX-KG (TaKaRa). The pGEX-KG/NP expression vector was used to transform BL21(DE3) *E. coli*, and expression of the GST-NP protein was induced by adding isopropyl-b-D-thiogalactoside (IPTG). Proteins were purified on a GST-affinity column and then dialyzed in phosphate-buffered saline (PBS) for 3 days [14].

2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (WB)

SDS-PAGE analysis was performed to confirm the expression of the target protein. To determine the antigenicity of the expressed NP protein, Western-blot analyses were performed. The primary antibody was serum obtained from a H9N2 swine influenza virus-immunized pig. Goat-anti-swine IgG(immunoglobulin G) conjugated with horseradish peroxidase (HRP) (Southern Biotech Associates Inc.) was used as the secondary antibody. The membrane was developed by incubation with 3,3′-diaminobenzidine (DAB) and hydrogen peroxide until the color developed sufficiently.

2.7. LAT

The optimal antigen concentration for sensitizing latex beads and the concentration of BSA in the blocking buffer were determined by the following processes. Latex beads (0.7 μm, Kexin Company, Shanghai, China) were washed three times with 0.1 M carbonate buffer (pH 9.6) and 0.02 M phosphate buffer (pH 4.5) respectively. The bead was then suspended in 2% ethyl-dimethyl-amino-propyl carbodiimide (EDAC) and incubated at room temperature for 4 h and centrifuged at 5000 ×*g* for 10 min. The supernatant was carefully aspirated out. After washing another three times with 0.01 M boric acid buffer (pH 8.4), the antigen which was serially diluted twofold with PBS (10 mmol L^{-1}; pH 7.4) from 1:2 to 1:1024 was added and incubated for 4–5 h to sensitize latex beads by passive adsorption. To block nonspecific binding, the beads were blocked with 0.1 0.5, 1.0, 1.5% bovine serum albumin (BSA) for 30 min at 37 °C and centrifuged. Then blocked beads were suspended in a storage buffer [5% glycerol, and 0.1% sodium azide (NaN_3) in phosphate buffered solution (PBS); pH 7.4] and used to react with either PBS (1:1) or anti-H9N2-positive serum to determine the optimal antigen concentration and the concentration of BSA in the blocking buffer (matrix). In detail, a 15-μL aliquot of sensitized beads was mixed with a 15-μL aliquot of PBS or anti-H9N2-positive serum, stirred gently, and observed for any agglutination reaction within 5 min. Test results were scored as rapid agglutination of 100% of sensitized latex beads with obvious ring formation (++++); agglutination of 75% of sensitized latex beads with some level of ring formation (+++); agglutination of >50% of sensitized latex beads but with no ring formation (++); fine-particle agglutination, usually involving <25% of sensitized latex beads and interpretation questionable (+); and no visible agglutination greater than that of the negative control sample (−). Agglutination reactions + to ++++ were considered positive results.

2.8. Specificity and reproducibility of the LAT

To determine the specificity of the LAT, the cross-reaction was evaluated with anti-H3N2-positive sera, anti-H1N1-positive sera, and anti-H9N2-positive sera and with serum samples positive for