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Development of a convenient immunochromatographic strip for the diagnosis of infection with Japanese encephalitis virus in swine

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A B S T R A C T

Japanese encephalitis (JE) is caused by the Japanese encephalitis virus (JEV). It is a major public health problem in Asia. JEV infects swine which results in fatal encephalitis, abortion and stillbirth in pregnant sow, and hypospermia in boars. Swine is a viral amplifier, and thus plays a critical role in JEV transmission. Thus, development of a rapid method for JEV detection in swine is required for clinical JE diagnosis, as well as to suppress viral spread. In this study, a convenient and rapid immunochromatographic strip (ICS) was developed for detecting JEV in swine using two monoclonal antibodies (MAbs) (2A2 and 4D1) against the E protein of JEV. Results showed that colloidal gold-conjugated MAbs 2A2 (CG-MAb) bond with JEV and the resulting complex was held by the other MAb 4D1 at the test line to give a reddish-purple band. Sensitivity tests demonstrated that ICS can detect 2.5×10^5 PFU of JEV. The clinical screening results showed that the specificity and sensitivity of the ICS were 99.3% and 85.7% respectively as compared to that of RT-PCR. This suggests that the MAbs-based ICS test can be used as a convenient method for the rapid detection of JEV in infected swine samples.

1. Introduction

Japanese encephalitis (JE) is caused by the Japanese encephalitis virus (JEV). It threatens public health and has direct impact on animal husbandry in southern and eastern Asia. Although JEV is primarily associated with JE in human specimen, it is also an important pathogen in swine. It has serious consequences in sows reproduction and death in piglets (World Health Organization, 1998; Van den Hurk et al., 2009). Furthermore, pigs are the main hosts of JEV from which infected mosquitoes transmit the virus to humans. JEV belongs to the *Flaviviridae* family. It contains a single positive 11-kb RNA genome with three structural proteins, designated as capsid (C), membrane (M) and envelope (E), as well as seven nonstructural proteins, designated as: NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5 (Chambers et al., 1990; Sumiyoshi et al., 1987). The E protein is a major immunogenic protein of JEV that induces neutralizing antibodies and is recognized as a protein candidate for vaccines and for the development of diagnosis method.

Several laboratory methods have been developed for the detection of JEV antibodies or antigens in swine, such as RT-PCR (Paranjpe and Banerjee, 1998), real-time PCR (Huang et al., 2004), neutralization test (Ting et al., 2004), enzyme-linked immunosorbent assay (ELISA) (Yang et al., 2006; Jia et al., 2002, 2005) and dot enzyme immunoassay (Cardosa et al., 1993). However, the application of these assays is limited by their requirements of laboratory operations, skilled technicians and special equipment/facilities. Therefore, the development of a rapid, specific and easily performed assay is crucial for the rapid detection and surveillance of JEV infection in swine.

The immunochromatographic assay is recognized as a new technique in which a cellulose membrane is used as the carrier and a colloidal gold-labeled antigen or antibody is used as the tracer. This method has been used widely for the diagnosis of many contagious human diseases. Recently, it has been effectively used to detect some animal viruses, such as porcine reproductive and respiratory syndrome virus (PRRSV) (Magar et al., 1993), bovine virus diarrhea and white spot syndrome viruses (Kameyama et al., 2006; Sithigorngul et al., 2006), yellow head virus (YHV) (Sithigorngul et al., 2007), streptococcus suis type 2 (SS2) (Yang et al., 2007) and avian influenza virus (AIV) (Peng et al., 2008).

In this study, MAbs (2A2 and 4D1) against the envelope (E) protein of JEV were generated and used to develop an immunochromatographic strip (ICS) test for the rapid detection of JEV in swine.

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The sensitivity and specificity of ICS test were evaluated by comparing with the conventional RT-PCR. Subsequently, the ICS test was used to detect JEV in 188 suspected swine samples. The results of the RT-PCR and that of the ICS tests were not significantly different ($\kappa = 0.889$).

2. Materials and methods

2.1. Cells, virus and animals

BHK-21 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 10% heated-inactivated fetal bovine serum (FBS), 100 μg/ml streptomycin and 100 IU/ml penicillin at 37 °C with 5% CO_2. Murine myeloma cell SP2/0 (8-Ag14) was stored in the laboratory and cultured in RPMI 1640 (Sigma–Aldrich, MO) supplemented with 10% heated-inactivated FBS, 100 μg/ml streptomycin and 100 IU/ml penicillin at 37 °C with 5% CO_2. JEV (strains P3 and SA14-14-2) was produced and tittered in BHK-21 cells. Unless otherwise specified, JEV refers to JEV strain SA14-14-2. Pseudorabies virus (PRV, Ea strain), porcine reproductive and respiratory syndrome virus (PRRSV, YA strain), porcine circovirus type 2 (PCV-2, Yu-A strain), foot and mouth disease virus (FMDV, O/ES/2003 strain) and classical swine fever virus (CSFV, CWH strain) were stored in the laboratory.

2.2. Preparation of E protein

The E gene fragment was amplified from JEV-infected BHK-21 cells by a one-step RT-PCR, with special primers (forward: 5′-CTTAGATCTATGGGCAATCGTGACTTCATA-3′, reverse: 5′-CTTGGATCCCACATTGGTCGCTAAGAACAC-3′). Subsequently, the target fragment was cloned into the pGEX-KG vector and fused at the N terminal of GST (glutathione S-transferase). The recombinant plasmid, named pKG-E, and the control plasmid (pGEX-KG) were then transformed into competent *Escherichia coli* BL21 cells and induced with isopropyl-dthiogalacto-pyranoside (IPTG). After centrifugation (4000 × *g*, 10 min), the bacterial pellet was suspended and sonicated until a clear lysate was obtained. The target protein (GST-E) or the labeling protein (GST) was purified with a commercial protein purification product (SephadexG-200, Pharmacia) and divided into small aliquots of 2 mg/ml concentration then stored at −80 °C.

2.3. Preparation of monoclonal antibodies against E protein

The MAbs against the E protein were produced as previously described (Huang et al., 2007). In brief, 5-week-old female SPF BALB/c mice were immunized subcutaneously with 100 μg of the recombinant protein GST-E at 2-week intervals. Four weeks after the last booster and 3 days before cell fusion, the mice were boosted with 40 μg of the GST-E. Three days later, mice splenocytes were harvested and fused with SP2/0 using 50% polyethylene glycol (Sigma–Aldrich, MO). Hybridoma culture supernatants were screened using ELISA. The positive hybridoma cells were cloned by a limiting dilution and the stable hybridoma clones were injected into liquid paraffin-pretreated abdominal cavities of BALB/c mice. Subsequently, the MAbs were harvested and purified from the seroperitoneum with an antibody purification kit according to the manufacturer's specifications (NAb™ Protein A/G Spin Kit, Thermo Scientific, USA). MAbs activity was characterized by Western blot and indirect immunofluorescence assay (IFA).

2.4. Western blot and indirect immunofluorescence assay (IFA)

For Western blot analysis, 100 μg GST-E or GST purified protein was loaded on 10% SDS-PAGE. Separated proteins were electro blotted onto a nitrocellulose membrane. The nonspecific antibody-binding sites were blocked with 1% bovine serum albumin (BSA) in TBS–T buffer (10 mM Tris–HCl pH 8.0, 150 mM NaCl, and 0.05% Tween-20) then membranes were reacted with MAbs (2A2 or 4D1). The resulting blot was treated with peroxidase-conjugated goat anti-mouse IgG (SouthernBiotech, USA). 3,3-Diaminobenzidine tetra hydrochloride (DAB) was used as the substrate for membrane development.

For IFA, BHK-21 cells were seeded into six-well tissue culture plates (Costar Corning Inc., Corning, NY) at 2.5×10^5 concentration of cells/well. When the cells reached approximately 70–80% confluence, the culture medium was removed. The cells were washed three times with PBS (pH 7.4) and incubated with pre-chilled DMEM containing 2.5×10^5 PFU of JEV for 1 h at 37 °C. After removing the virus, fresh medium was added and cultures were incubated at 37 °C. At 72 h post-infection, the cells were fixed with absolute methanol and processed for indirect immunofluorescence assay (IFA) using MAbs (2A2 or 4D1), followed by fluorescein isocyanate-conjugated goat anti-mouse IgG. Finally, fluorescent images were examined under a fluorescent microscope.

2.5. ELISA additive tests

An additive index (AI), which compared the ODs obtained by two MAbs assayed under standardized conditions, either alone or in a mix was calculated for each pair of MAbs (Friguet et al., 1983) using the formula: $AI = \{[2 \times A_{1+2}/(A_1 + A_2)] - 1\} \times 100$, where A_1 and A_2 were the ODs obtained when the MAbs were assayed separately, and A_{1+2} was the OD when the same amounts of the two MAbs were pooled in the same well. Provided the concentrations of the MAbs were saturated for the purified GST-E protein, the AI would be negligible if both MAbs were detected at the same epitope and close to 100 when the two epitopes were topographically unrelated (Huang et al., 2007). The lowest AI reported for MAbs at different epitopes on JEV was considered as the threshold for evaluating epitopic correlation.

2.6. Preparation of colloidal gold-MAb conjugate and immunochromatographic strip (ICS)

15-nm diameter colloidal gold particles were prepared and mixed with MAb 2A2 (CG-MAb) as Zhang et al. (2006) described. The immunochromatographic strip composed four components, a sample pad, a conjugate pad, an absorbent pad (Jiening Bio, Inc., Shanghai, China) and a nitrocellulose membrane (Whatman, Dassel, Germany) as illustrated in Fig. 1. The CG-MAb solution was dispensed onto glass fiber paper (conjugate pad) (300 mm × 6 mm) at a rate of 50 μl per cm (about 2 μg/cm) using an XYZ3050 Dispense Workstation (BioDot, Inc., Sky Park, Irvine, CA) dried under vacuum. MAb 4D1 (2 mg/ml) and goat anti-mouse IgG (2 mg/ml) were dispensed at the test or the control line on the nitrocellulose membrane using an XYZ3050 Dispense Workstation. After drying for 2 h at 37 °C, the membrane strips were blocked by incubating with PBS (pH 7.4) containing 2% (w/v) nonfat dried milk for 30 min and washed three times with PBS containing 0.1% (v/v) Tween-20 for 3 min each time. The membrane was dried for 2 h at 37 °C and stored at 4 °C. The sample pad, conjugate pad, immobilized nitrocellulose membrane and absorbent pad were glued together on a backing plate (300 mm × 70 mm) and then cut into 3-mm-wide strips using a CM-4000 cutter (BioDot, Inc., Sky Park, Irvine, CA). The dried strips were stored at 4 °C.

2.7. Detection principle and test procedure

During the test, samples that reacted with the CG-MAb conjugate to form a CG-MAb–JEV complex which flowed laterally onto