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 sows, and hypospermia in boars. Swine is a viral amplifier, and thus plays a critical role in JEV transmission. Therefore, 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 MAb 2A2 (CG-MAb) bond against the E protein of JEV. Results showed that colloidal gold-conjugated MAb 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 color. The ICS 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 MAb-based ICS test can be used as a convenient method for the rapid detection of JEV in infected swine samples.

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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 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 (HIV) (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 (κ = 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% CO₂. 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% CO₂. JEV (strains P3 and SA14-14-2) was produced and titered 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′-CTTAGATCTTAGCCACATCGATCTGCA-3′, reverse: 5′-CCTGATCCTGCGGTGCTGCAATTACAC-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-d-thiogalacto-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 from a commerical 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 hybridomas were cloned in 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 serumoprotein 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 electroblotted 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 × 10⁵ 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 × 10⁵ 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 × A₁A₂)/(A₁ + A₂)] − 1 × 100, where A₁ and A₂ were the ODs obtained when the MAbs were assayed separately, and A₁A₂ 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 epitope 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
the nitrocellulose membrane by capillary action. For a positive sample, the specific antibody bonded with the JEV, forming a red band at the test region. Excess CG–MAb conjugate and bond with goat anti-mouse IgG at the control line, forming another red band. As a procedural control, the red band at the control line would appear regardless of the presence of specific antibody. Thus, the appearance of two red bands in the read-out zone indicates a positive result and the appearance of only one red band at the control line indicates a negative result. The ICS was laid on a flat bench and 100 μl crudely purified sample added to the sample hole and the result was obtained within 5 min.

2.8. Specificity and sensitivity of the ICS test

To evaluate the specificity of the ICS test, several different viruses associated with swine diseases such as PRV, PRRSV, PCV-2, FMDV and CSFV were tested. JEV wild strain (P3 strain) and live-attenuated SA14-14-2 vaccine strain produced in BHK-21 cells were used as positive controls, while PBS solution and health control were used as negative controls.

For sensitivity evaluation, JEV stock ($2 \times 10^6$ PFU) was diluted 2-fold continuously with PBS. Each dilution was then applied to the ICS test. The sensitivity was determined by finding the end point dilution. To compare the sensitivity between ICS test and RT-PCR, the diluted JEV stock was evaluated by RT-PCR with a pair of primers (forward: 5′-AGAGTCGACAAACAATGTAAATGAGAAAATGCA-3′ and reverse: 5′-ACTGCGGCCGCTGTTCTTCCTACCTACGCTACATAC-3′) for 561-bp DNA fragment. The sensitivity of RT-PCR was evaluated by determining the end point dilution.

2.9. Diagnosis of JEV infection in the field

188 clinical swine samples collected by the Veterinary Hospital at Huazhong Agricultural University were used for the detection of JEV. Five grams of brain/spleen/mummy fetus tissue in 10 ml of sterile physiological saline were homogenized in a sterile grinder and centrifuged at 1000 $\times$ g for 20 min. The supernatant was filtered with 0.22 μm filter membrane and the percolates collected as tissue samples (Yang et al., 2007). All samples were tested by ICS test and RT-PCR, as described earlier. The significant difference between the ICS test and RT-PCR was tested using the $\kappa$ statistical analysis (Donner and Klar, 1996) method.

3. Results

3.1. Production of monoclonal antibodies for JEV

Five hybridoma clones (HC) secreting MAbs and JEV (E) protein were screened by a limiting dilution and indirect ELISA. Two of the five HC showing high titers (data not shown), 2A2 and 4D1 were applied to a Western blot and IFA to identify their specificity for the E protein and JEV. As shown in Fig. 2A, both 2A2 and 4D1 can specifically detect recombinant protein GST-E (79KD) but not GST (26KD). In addition, as shown in Fig. 2B, a strong fluorescent signal was detected in the JEV-infected BHK-21 cells by the IFA test when 2A2 and 4D1 were used as primary antibodies. The result of the ELISA additive test showed that the AI value between 2A2 and 4D1 was about 100, implying that the two MAbs were on different epitopes (Table 1). This indicates that MAbs 2A2 and 4D1 may be used as specific antibodies to detect JEV in the ICS test.

3.2. Specificity of the ICS test

To determine the specificity of the ICS, several viruses associated with popular viral diseases in swine production were used as nonspecific virus samples. All the samples were tested by JEV conventional RT-PCR. As shown in Table 2, negative results were obtained from the samples of PRV, PRRSV, PCV-2, FMDV, and CSFV stock, while positive reactions were only observed in the samples of JEV P3 and JEV SA14-14-2. The results showed that >90% of the JEV samples can be tested by the ICS. The results are consistent with the results of the RT-PCR, suggesting that the ICS test has high specificity for JEV. In addition, the plaque-forming assay verified that the viral copies in the two samples which were positive with RT-PCR but negative with ICS test were under the threshold of the

| Table 1 |

<p>| Analysis of epitopes defined by MAbs against the E protein of JEV. |
|-----------------|------|------|</p>
<table>
<thead>
<tr>
<th>MAb</th>
<th>2A2</th>
<th>4D1</th>
</tr>
</thead>
<tbody>
<tr>
<td>2A2</td>
<td>$2^a$</td>
<td>104</td>
</tr>
<tr>
<td>4D1</td>
<td>5</td>
<td>7</td>
</tr>
</tbody>
</table>

| Additivity index. |
|-----------------|------|------|
| a Additivity index. |
| b Standard deviation. |
ICs assay (data not shown), so the failure to detect the two positive samples maybe attributed to the sensitivity of the assay.

3.3. Sensitivity of the ICS Test

$2 \times 10^6$ PFU and $2 \times 10^4$ PFU of JEV were serially diluted with PBS and tested by ICS and RT-PCR respectively. The results showed that the minimal amounts that could be tested by ICS and RT-PCR were approximately $2.5 \times 10^5$ PFU and $3.2 \times 10^2$ PFU, respectively (Fig. 3A and B). This demonstrated that the ICS test was slightly less sensitive than RT-PCR.

3.4. Clinical samples detection

The RT-PCR results of 188 clinical samples from the pigs suspected of JEV infection showed 42 positive and 146 negative antigens. Whereas the ICS test results showed 37 positive and 151 negative antigens. The virus and the sequence for detecting JEV were confirmed previously so the sequence analysis on these clinical samples was not conducted. Thus, the specificity and sensitivity of the ICS and the RT-PCR, were 99.3% and 85.7%, respectively. Therefore, there was no significant difference ($\chi = 0.889$) between the ICS test and RT-PCR (Table 3).

4. Discussion

JEV is an important pathogen that causes Japanese encephalitis a reproductive disorder in pigs. JEV threatens human health with an estimated worldwide annual incidence of 50,000 human cases and 10,000 deaths (World Health Organization, 1998; Van den Hurk et al., 2009). Thus accurate diagnosis of the responsible pathogen of suspected encephalitis infections is important for optimal patient management. However, the application of conventional assays, such as RT-PCR and viral isolation is limited by their requirements of laboratory operations, skilled technicians and special equipment/facilities. Therefore, there is an urgent need to develop a convenient and rapid method to detect JEV.

Gold conjugate techniques have been used increasingly in ICS for rapid disease diagnosis. The ICS test kits were developed in this lab...
oratory and applied in farms for detecting Streptococcus suis type 2 (SS2) and AIV (Yang et al., 2007; Peng et al., 2008). Other experiments facilitated the development of an ICS test to detect JEV. First, MAbs against the immunogenic E protein of JEV (Chen et al., 2005), the main envelope protein displayed on the viral particle was generated. In order to generate adequate immunogen replacing JEV, the recombinant plasmid pKG-E was constructed and used to prepare fusogenic protein GST-E. Five MAbs specific to the JEV envelope protein were produced from five stable hybridoma cell lines which were generated by the fusion of murine SP2/0 with splenocytes isolated from a BALB/c mouse immunized with recombinant protein GST-E. ELISA, Western blot and IFA were used to select positive hybridoma cell clones which constitutively produced MAbs with high reactionogenicity for JEV. Secondly, in order to detect JEV with the ICS, the epitopes of two selected high-affinity MAbs should not be related. So the ELISA additive test was used to detect whether or not the epitopes detected by the different MAbs were overlapping (Friguet et al., 1983). The Als calculated pairs of neutralizing MAbs suggested that the epitopes detected by the MAbs (2A2 and 4D1) were reciprocally separated (Table 1).

Currently, there are several assays available for the detection of JEV. The JEV TaqMan assay could detect all seven tested strains of JEV but provided negative results for the other nine flaviviruses and encephalitis viruses are proven. In addition, for cultured JE virus, less than 40 plaque-forming unit (PFU)/ml of virus load can be detected by TaqMan RT-PCR (Huang et al., 2004) which shows greater sensitivity and specificity than traditional RT-PCR methods. In the ICS specificity test, CSVF, which is also a member of Flaviviridae family, was used to examine the cross-reaction because other members of Flaviviridae may produce false positive results for the affinis structure (De Madrid and Porterfield, 1974). Eight positive CSVF samples showed negative results when tested with the strip. A sample from the Flaviviruses family, such as dengue virus, west nile virus or yellow fever virus were needed to further verify the specificity of the ICS test in our subsequent study. Although the sensitivity of the ICS test developed was slightly less than that of RT-PCR it has some merits such as it can be used on-site for rapid detection of JEV and the results can be visually seen within 5 min. This assay is easy to operate and can be performed by farmers. In comparison, conventional RT-PCR and TaqMan RT-PCR are labor-intensive and time-consuming; require several freshly prepared controls for standardization, probes and dyes, thus making them unsuitable for the rapid and on-site characterization of JEV infection.

However, some samples lightly infected with JEV had negative results with the strip and positive results by RT-PCR. Thus the strip test is not recommended for screening of lightly infected stock or some potential carriers of JEV but suitable for farm confirmation of severe JEV infection such as in moribund swine just prior to and during a full-blown JEV outbreak. It is necessary that the suspected samples without typical signs of JEV infection be further confirmed by histological, immunological, or molecular tests.

Yang et al. (2007) noted that the pH of the gold solution and the quantity of the MAbs are the two most important factors for the successful preparation of a gold–antibody conjugate. The gold solution bind antibody more effectively when its pH is close to 0.5 or higher than the isoelectric point (pl) of the MAb (the pl of 2A2 is about 8.1). Tighter binding occurs at a higher pH, however it may have a denaturing effect on the antibody, resulting in a less effective probe. Having excess antibody coupled with gold particles, may make the probe less effective because some of the weakly bound antibody may detach from the particles and the free antibody will compete for binding sites with the gold–labeled antibody. In addition, labeled CG will decrease the sensitivity. Since both the specific and nonspecific components in the samples bind to the CG, adequately labeled CG-MAb solution should be dispensed onto the glass fiber paper (conjugate pad) to avoid minimizing the sites available for specific virus.

In summary, an ICS test based on two MAbs (2A2 and 4D1) against two different antigenic sites of E protein was developed for the detection of JEV. With RT-PCR as a reference, this test has good specificity (99.3%) and sensitivity (85.7%). The results obtained by RT-PCR and the ICS tests agreed (κ = 0.889). Though the ICS test was less sensitive than that of RT-PCR, it can be used as a convenient and rapid tool for detecting and controlling an outbreak and spread of JE.

Conflict of interest

The authors declare that they have no conflicts of interest.

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