Competitive ELISA for the Detection of Antibodies to Rift Valley Fever Virus in Goats and Cattle

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ABSTRACT. Rift Valley fever virus (RVFV) is one of the important emerging viral diseases of serious impact in public health and animal hygiene both in human and animal industries. In this study, we developed a monoclonal antibody-based competitive ELISA for the detection of antibodies to RVFV in goats and cattle. The recombinant N protein of RVFV was expressed in E. coli with a six-histidine tag, and the purified N protein was used for detecting antigen with a competitive monoclonal antibody against RVFV antibodies. The competitive ELISA (C-ELISA) could detect antibodies at 9–11 days after inoculation in goats and cattle with a sensitivity of 94.7% (virus neutralization titer >32) and specificity of 99.7%, respectively. In addition, the C-ELISA did not show any cross-reactivity with positive sera against arboviruses such as Akabane, Aino, Chuzan, Ibaraki and bovine ephemeral fever virus, which are prevalent viral agents in ruminant animals throughout Southeast Asia. The results of the present study indicate that the C-ELISA is a simple, rapid and convenient serodiagnostic method for RVFV in goats and cattle.

KEY WORDS: arbovirus, competitive ELISA, recombinant N protein, Rift Valley fever virus.


Rift Valley fever virus (RVFV) is a zoonotic, mosquito-borne member of the genus Phlebovirus in the family Bunyaviridae that is transmitted by Aedes and Culex species. RVFV causes high mortality in newborn kids and abortions in pregnant sheep, goats and cattle [1, 23]. It was first identified in sheep by Daubney et al. in Kenya in 1931 [6] and was not reported outside sub-Saharan countries until 1977, when it suddenly emerged to cause a large outbreak in Egypt. However, the recent first outbreak in the Arabian Peninsula (Yemen and Saudi Arabia) outside Africa and re-emergence of the disease in East Africa are of global medical and veterinary concerns [2, 9]. Global warming, which facilitates spread of mosquito-borne viruses, has raised concerns that other regions of the world could be receptive to the virus [18]. Accurate diagnosis of RVF can be achieved by serological tests in combination with clinical observation and epidemiological history [7]. Classical methods for the detection of antibodies to RVFV are hemagglutination inhibition, complement fixation, virus neutralization (VN) test, and immunofluorescence assay (IFA) [24]. Disadvantages of these tests include the health risk to laboratory personnel by manipulating live virus, and so restrictions concerning their use outside RVF endemic areas [13]. Therefore, these inconveniences led to development of a safe and reliable tool for rapid diagnosis of RVF [18]. Different types of enzyme-linked immunosorbent assays (ELISA) have been recently developed and evaluated for serodiagnosis of RVF in humans and animals. A highly sensitive and specific indirect ELISA based on the use of β-propiolactone inactivated and/or gamma-irradiated, sucrose-acetone extracted antigens derived from tissue culture or mouse brain for the detection of IgG and IgM antibodies to RVFV in domestic and wild animals and humans has been developed and validated [13, 14, 16, 17].

In this paper, we describe development of the competitive ELISA (C-ELISA) based on recombinant nucleocapsid protein (rNp) and monoclonal antibodies (MAb) for the detection of antibodies in cattle and goats.

MATERIALS AND METHODS

Virus and cells: The RVFV Smithburn strain was obtained from South Africa (OBP, Onderstepoort, South Africa) and used as a reference virus. Vero cells were cultured with alpha minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA, U.S.A.) and antibiotic-antimycotic solution (Invitrogen). A VN test was carried out using 96-well tissue culture microplates with flat-bottomed wells seeded with 3 × 10⁵ Vero cells per well according to the manual of standards for diagnostic tests of OIE [12]. The titer was expressed as the reciprocal of the serum dilution that inhibited >75% of the viral cytopathic effect (CPE). A serum with a titer of >log_{10} 0.6 was considered positive.

Expression and purification of the N protein of RVFV: The N protein of RVFV was synthesized (Bioneer, Daejeon, Korea) on the basis of sequence information for the RVFV Smithburn strain (NCBI Accession number: DQ380157) [12]. The 738 bp gene was cloned into pCR 4 TOPO (Invit-
rojen). The N protein gene was further amplified by PCR, and restriction sites were introduced using RVF1 forward (5’-GCA TGG ATG GAC AAC TAT CAA GAG-3’) and reverse (5’-GGT ACC TTA GGC TGC TGT CTT-3’) primers. The PCR product was then digested with Sph I and Kpn I (underlined in primer sequence) and ligated into the vector pGemT-easy (Promega, Madison, WI, U.S.A.). The rNp was expressed with a six-histidine tag using a QAexpress system (Qiagen, Duesseldorf, Germany). The protein concentration was determined using a BCA Protein Assay system (Qiagen, Duesseldorf, Germany). The protein concentration was determined using a BCA Protein Assay system (Qiagen, Duesseldorf, Germany).

Monoclonal antibodies: MAbs were produced by the cell fusion method as described previously [4]. Two 8-week-old BALB/c female mice were inoculated with 100 µg of the rNp via the footpad. The mice were euthanized 2 weeks after injection, and their popliteal lymph nodes were extracted. Lymphocytes were fused with SP2/0 myeloma cells by using polyethylene glycol 1500 (50% sol.). Hybridoma cells were screened by indirect ELISA and further analyzed by Western blotting. Their specificity was determined by IFA. MABs were also applied to RVF I-ELISA (BDSL) instead of mouse anti-RVF antibody to determine the reactivity to RVFV. Finally, MAB was isotypes with the manufacturer’s instructions and stored at –20° C until use.

Hyperimmune sera from goats: Purified rNp (1 mg/ml) was prepared and mixed with the same volume of Freund’s incomplete adjuvant for production of hyperimmune anti-rNp antibodies. Two goats (10 months) were immunized subcutaneously with the rNp three times with an interval of two weeks. Before booster injection, a blood sample was taken and tested by indirect ELISA [5]. Whole sera were collected at 6 weeks after primary injection.

Positive sera from goats, cattle and sheep: Since RVFV is an exotic disease in Korea, the animal experiment was conducted in biosafety level 3 (BL3) laboratory at the National Veterinary Research and Quarantine Service (NVQRS). Two 1-year-old Holstein cattle and five 10-month-old goats were inoculated subcutaneously with 1 ml of the live-attenuated Smithburn strain of RVFV [8]. A total of 105 blood samples were collected at an interval of 2 or 3 days, and sera were used for VN tests and C-ELISA. Animal experiments were conducted according to the guidelines of the NVQRS Animal Ethics Committee (Approval number: 2008–38).

Reference positive and negative sheep sera from a commercially available I-ELISA kit (BDSL, UK) were also tested for comparative validation of the C-ELISA in this study.

Negative sera and mosquito-borne disease-positive sera: Normal goat (n=400) and cattle (n=600) sera were collected from domestic livestock throughout the country.

A total of 96 mosquito-borne disease-positive sera were also tested for cross-reactivity in the C-ELISA. All sera were tested by VN and confirmed positive to bovine mosquito-borne viral diseases from NVRQS [10].

There were 54 (56.3), 26 (27.1), 59 (61.5), 29 (30.2) and 93 (96.9) positive cases (%) of Akabane, Aino, bovine ephemeral fever and Ibaraki virus, respectively. In addition, although 96 sera were further examined for the presence of antibodies against bluetongue virus by using a Bluetongue Virus Antibody Test Kit (VMRD, Pullman, WA, U.S.A.), all the sera turned out to be negative in this experiment.

C-ELISA: To set up the optimal conditions for the C-ELISA, standard checkerboard titration of the rNp and MAB was carried out [5]. ELISA plates (MaxiSorp™, Nunc, Roskilde, Denmark) were coated with 1 mg/ml of the rNp in 0.01 M phosphate buffered saline (PBS) (pH 7.4) in a volume of 50 µl per well and incubated at 37°C for 1 hr. Plates were washed three times with PBS containing 0.05% tween 20 (PBSST). Following washing, 50 µl of each test and control sera diluted 1:10 in diluents (5% skim milk in PBS) were added to the plate. Strong positive, weak positive and negative control sera from a commercial I-ELISA kit (BDSL) were also included as reference in each plate. The same volume of MAB (0.75 µg/ml) was added to wells. After incubation at 37°C for 1 hr, plates were washed, and 50 µl of goat anti-mouse IgG conjugated horseradish peroxidase (Thermo Fisher Scientific, Waltham, MA, U.S.A.) according to the manufacturer’s instructions and stored at –20° C until use.

Expression and purification of rNp of RVFV: When the rNp of RVFV was expressed in E. coli M15 cells, denaturation with 8 M urea was used for purification, mainly due to the insoluble characteristics of the rNp. The rNp of RVFV showed an apparent molecular mass of 30 kDa (N protein 27 kDa+histidine 3 kDa) in SDS-PAGE and Western blot using anti-RVFV mouse hyperimmune ascites and sheep polyclonal antibodies as shown in Fig. 1.

Production and characterization of MABs: A total of 10
MAbs showed positive reactivity against rNp of RVFV in indirect ELISA. Among the 10 MAbs, 4 were also positive with the I-ELISA (BDSL). One MAb, designated as MAb 1–59, was finally selected for the C-ELISA in this study. MAb 1–59 was an IgG2b isotype with a kappa light chain.

Optimization of the C-ELISA: Through checkerboard titration of the rNp and MAb, the optimal concentrations of the purified N protein and MAb 1–59 as a competing antibody were determined to be 1 µg and 0.75 µg, respectively. In addition, the optimal dilutions of the test and control goat sera against rNp were final dilutions of 1:10 and 1:4 in PBS, respectively.

The threshold cut-off value of PI was finally established at 31% using normal goat and cattle samples (n=1,000) by calculating mean PI plus 3 times the standard deviations to secure high specificity (Fig. 2). With this cut-off value, the C-ELISA showed 2 false positives from cattle sera and 1 false positive from goat sera, respectively. All together, the C-ELISA resulted in 3 false positive reactions out of 1,000 samples, showing 99.7% specificity for the VN test. In addition, the results of comparative evaluation using the reference sheep sera also showed an equivalent PI values both in the C-ELISA and I-ELISA (Fig. 3).

In addition, 96 mosquito-borne virus-positive sera were confirmed to be negative for bluetongue virus. For the validation of specificity, the C-ELISA was also tested with the 96 mosquito-borne disease-positive sera. As indicated in Fig. 4, the results were all negative in the C-ELISAs, supporting the specificity of the C-ELISA against other mosquito-borne virus-positive sera.

Comparison of diagnostic efficiency of the C-ELISA: For evaluation of performance of the C-ELISA, the C-ELISA was initially compared with the VN test as the gold standard by using a panel of sera from cattle and goats inoculated with live-attenuated Smithburn strain of RVFV. The relative sensitivity and specificity of the C-ELISA were also assessed with a commercially available I-ELISA, respectively.

When the performance of the C-ELISA was evaluated with the VN test, it was revealed that the neutralizing antibodies were detected between days 7 and 9 in experimentally inoculated cattle and goats. On the other hand, the C-ELISA could detect the positive signals between days 9 and 21 (Table 1 and Fig. 5). Although the C-ELISA could not detect all the positive sera with low VN titers (VNT <16), the relative sensitivity turned out to be 94.7% with the sera (VNT >32), as shown in Table 2.

DISCUSSION

RVF is one of the important zoonotic diseases in terms of the impact on public health and animal hygiene in both humans and livestock animals. In this respect, RVFV requires extensive monitoring by using reliable diagnostic
Fig. 4. The distribution of percent inhibition according to the competitive ELISA for arboviruses using 96 mosquito-borne disease-positive sera. The horizontal line represents the cut-off value.

(A) Cattle

(B) Goat

Fig. 5. Comparison of early antibody responses by C-ELISA and I-ELISA for sera of two cattle (A) and goats (B: n=5) inoculated with a live attenuated Smithburn strain of RVFV.
COMPETITIVE ELISA FOR THE DETECTION OF RVFV ANTIBODIES

At present, many diagnostic methods such as VN, hemagglutination inhibition and complement fixation are available for RVFV. However, all the protocols required the use of inactivated or live virus, thus posing the potential risk of virus release into the RVF-free environment. For this reason, we developed a C-ELISA based on rNp of RVFV and MAb for diagnosis. A main advantage of present approach is the development of a convenient diagnostic tool in a less laborious and expensive way with high safety in RVF-free countries.

In fact, previous studies have revealed that the N protein of RVFV is a highly conserved among RVFV strains [19–21, 26, 27]. In addition, it was also reported that N protein is the main immunodominant viral protein in other members of the Bunyaviridae family [22, 25]. Since rNp lacks infectivity with considerable stability, it is one of the suitable candidates for use as a diagnostic antigen in an ELISA [7, 8, 15, 18].

In this study, we also found that MAb for RVF N protein showed a considerable specificity by IFA, Western blot and ELISA through comparative evaluations (data not shown). During the last few years, a polyclonal antibody-based IgG-sandwich ELISA was developed [13, 14, 16], and this was followed by the production of RVFV-specific MAbs [16, 17, 19, 25]. Moreover, MAb against the N protein of RVFV produced specific and sensitive responses in IgM capture and IgG sandwich ELISA for human sera [28]. Recently, it was also reported that MAb generated by DNA immunization of RVFV is a useful tool the C-ELISA, especially in a broad range of species [11].

In the present study we produced positive panel sera in cattle and goats by inoculation of a live-attenuated Smithburn strain of RVFV, mainly due to limited reserves of positive sera from the field. Based on the kinetics of immunological responses in experimental animals with live attenuated RVFV, we conducted a comparative evaluation with a currently available commercial I-ELISA [16]. It was reported that I-ELISA based on rNp was more sensitive than the VN test for the detection of early antibodies in sheep inoculated with the Smithburn strain of RVFV [8, 16]. However, when we evaluated the efficiency of the C-ELISA by using the sera panel from goats and cattle inoculated with RVFV, the results indicated that the C-ELISA could detect early antibodies at 9–11 days post inoculation except in 1 goat, which required 2 days more than the VN test. The reason for this late detection in the goat was presumably due to the difference in species because the I-ELISA also showed similar results in this study.

Interestingly enough, the sensitivity of the C-ELISA was relatively lower with serum samples having low VN titers (<16); however, it showed 94.7% sensitivity with samples of higher VN titers (>32). On the other hand, the I-ELISA showed a relative sensitivity of 89.4% in the comparative analysis in the present study. Although the discrepancy of previous result in sensitivity of the I-ELISA is not clear in this study, one possible explanation may be the differences in antibodies recognized by each assay because the C-ELISA could detect only the competitive epitope of the rNp of RVFV, while the I-ELISA covers the whole range of antibodies against rNp of RVFV, respectively [8, 16].

Table 1. Comparison of virus neutralization, competitive ELISA and inhibition ELISA in detecting antibodies from cattle and goats infected with a live-attenuated Smithburn strain of RVFV

<table>
<thead>
<tr>
<th>Days post inoculation</th>
<th>Cattle VN</th>
<th>C-ELISA</th>
<th>I-ELISA</th>
<th>Goat VN</th>
<th>C-ELISA</th>
<th>I-ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
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<td>7</td>
<td>1/2</td>
<td>0/2</td>
<td>0/2</td>
<td>4/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>9</td>
<td>2/2</td>
<td>1/2</td>
<td>0/2</td>
<td>5/5</td>
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<tr>
<td>11</td>
<td>2/2</td>
<td>1/2</td>
<td></td>
<td>2/5</td>
<td>2/5</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>1/2</td>
<td></td>
<td></td>
<td>4/5</td>
<td></td>
<td>4/5</td>
</tr>
<tr>
<td>16</td>
<td>2/2</td>
<td></td>
<td></td>
<td>4/5</td>
<td></td>
<td>4/5</td>
</tr>
<tr>
<td>21</td>
<td>5/5</td>
<td></td>
<td></td>
<td>5/5</td>
<td></td>
<td>5/5</td>
</tr>
</tbody>
</table>

Table 2. Evaluation of the C-ELISA for panel sera from cattle and goats infected with a live-attenuated Smithburn strain of RVFV

<table>
<thead>
<tr>
<th>Titer</th>
<th>No. Sera</th>
<th>C-ELISA</th>
<th>I-ELISA</th>
</tr>
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<tbody>
<tr>
<td>4</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>7</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>16</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>32+</td>
<td>38</td>
<td>36</td>
<td>34</td>
</tr>
</tbody>
</table>

a) Virus neutralization test.
b) Competitive ELISA, developed in NVRQS.
c) Inhibition ELISA (BDSL, UK).
d) Number of sera confirmed positive in VNT.
Besides, it is worthy to note that the C-ELISA did not show any cross reaction against indigenous mosquito-borne viral diseases such as Akabane, Aino, Ibaraki, BEF and Chuzan, supporting the diagnostic specificity of the present C-ELISA. In practice, it is quite important that the C-ELISA could differentiate RVF from other mosquito-borne viral diseases such as Akabane and Chuzan, the incidences of which are relatively ubiquitous. Since RVF is an important zoonotic disease of public health importance, it is desirable to have various diagnostic methods with considerable sensitivity without the possible incidence of false-positive results. Recently, Cetre-Sossah et al. evaluated a commercial competitive ELISA in sera of domestic ruminants in France, an RVFV-free country. As a result, all species were negative with a higher than recommended cut-off PI. Therefore, this variation may be attributed to biology, origin and differences among animals [3]. In this respect, different diagnostic thresholds could be applied for the animals from different geographical origins or distinct ruminant species and breeds for optimal ELISA performances [19]. For this reason, the optimal cut-off for the developed C-ELISA will require evaluation in more ruminants and other regions because the current cut-off only applies to cattle and goats in our country.

Although the C-ELISA still needs to be further evaluated with a large number of sheep sera from the field because the sheep is a major host animal, at least it was demonstrated that the C-ELISA using the rNp and MAb would be a useful diagnostic method for the detection and differentiation of antibodies to RVFV and other mosquito-borne viral diseases in goats and cattle.

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REFERENCES


