Development of an ELISA Based on Fusion VP232-452 Antigen for Detecting Antibodies against Aleutian Mink Disease Virus

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Abstract: For detection Aleutian mink disease virus (AMDV) antibodies, an ELISA was developed using the recombinant VP2332-452 protein as an antigen. Counter immunoelectrophoresis (CIEP) was used as a reference test to compare the results of enzyme linked immunosorbent assay (ELISA) and western blotting (WB), the specificity and sensitivity of VP2332-452-ELISA was 97.9% and 97.3%, respectively, which were higher than those of WB. Therefore, this VP2332-452-ELISA might be a preferable method for detecting antibodies against AMDV.

Aleutian mink disease virus (AMDV), a member of the genus Amdovirus, subfamily Parovirinae, family Paroviridae, is a single-stranded DNA virus with a genome length of 4.8 kb (1, 2). The AMDV genome contains two major open reading frames (ORFs), a 5’ ORF encoding the capsid proteins VP1 and VP2, and a 3’ ORF encoding the nonstructural proteins NS1 and NS2. Identified strains varied in pathogenicity from nonpathogenic (AMDV-G) to highly pathogenic (AMDV-Utah 1, AMDV-United, and AMDV-K) (3-5).

AMDV has been found in all mink breeding countries and causes the greatest financial loss to farmers, including decreased production, loss of breeding animals, and low-quality fur (6). This nonenveloped DNA virus is persistent in the environment and resistant to various physical and chemical treatments. AMDV infection is normally persistent and fatal, and there is no effective vaccine against the disease (7-9). Transmission of AMDV occurs horizontally by direct and indirect contact and vertically from pregnant mink to kits. Currently, the most successful strategies for eradicating AMD program are based on serological screening and culling of all antibody-positive animals.

AMD diagnosis is primarily based on the clinical signs and detection of AMDV antibodies. Counter immunoelectrophoresis (CIEP) has been used widely for routine detection of AMDV antibodies (10). Concerning antibody detection, ELISA has the advantage of being easily automated whereas the CIEP analysis is labor intensive with many manual tasks, requires sophisticated instruments, and not suited for automation. Currently, ELISA based on the baculovirus expressed whole VP2 protein has been developed, and it seems to be in good agreement with CIEP analysis (11). However, this ELISA has the disadvantage of requiring propagation and purification of large quantities of VP2 protein in eukaryotic baculovirus systems.
and expensive processes. Compared to eukaryotic expression system, the propagation and purification of the proteins are easier, and the cost is lower in *Escherichia coli* (*E. coli*) expression system. *E. coli*-expressed protein-based ELISA tests have a high sensitivity and specificity because of the high concentration of immunoreactive antigens and have been used widely for large scale surveys (12-15). However, the cost-effective ELISA based on *E. coli*-expressed protein for rapid detecting antibodies against AMDV has not been developed. Therefore, in present study, we developed a rapid, cheap, and sensitive ELISA base on *E. coli*-expressed proteins for screening antibodies against AMDV.

Bloom et al., (16) demonstrated that the antigenic regions or the most consistently immunoreactive regions were pVP2e, pVP2f, and pVP2g (between amino acids (aa) 290 and 525), which encompassed the analogs or surface loops 3 and 4 of canine parvovirus (CPV);(17, 18), containing the linear epitopes located on the external surface of the AMDV virion capsid proteins. These pVP2e, pVP2f, and pVP2g segments of capsid proteins of AMDV could be detected with all sera of mink infected with different AMDVs (ADV-TR, ADV-Utah, or ADV-Pullman). Moreover, Bloom et al., (19) identified one most important surface-exposed residues, 19 amino acids peptide VP2428-446 on VP2 segment. To express central antigen fragments of VP2 protein, the potential major hydrophilic region or antigenic peak on VP2 was calculated, which located in the central region from amino acids 332 to 452 (data not shown) by using DNASTAR LASERGENE software 99 (20). Primers VP2eF994: 5’TATGGATCCAGACCTAGGCACACTTTGGGGTG3’ (underlined nucleotides represent the *BamHI* site) and VP2gR1633:5’TATCTGCAGTTAAGCGTTGTTGCTGCTAGGT3’ (underlined nucleotides represent the *PstI* site) corresponding to this region (between aa 332 and 452) were designed. The amplified PCR product was sequenced, resulting in the expected size of 362 bp. The PCR product was cloned into the *BamHI*-*PstI* sites of pMAL-c2x vector (New England Biolabs). The correct orientation of inserts was confirmed by restriction analysis and nucleotide sequencing. The recombinant pMAL-VP2332-452 plasmid were transfected into BL21 *E. coli* (pLysS, Invitrogen). The expressed VP2332-452 fusion proteins in cell debris and supernatant were purified by using a HisPur™ Cobalt Purification Kit (Thermo Scientific) (Qiagen, Valencia, CA, USA) and then analyzed by SDS-PAGE and western blotting. Nitrocellulose membranes were probed with AMDV-positive mink sera (diluted 1:100) and phosphatase-labeled goat anti-feline IgG conjugates (1:2000
dilution) (Thermo Scientific). SDS-PAGE showed the VP2332-452 fusion protein with an approximate molecular mass of 64 kDa (Fig. 1A), which is consistent with expected size of fusion protein (24.5 kDa protein VP2332-452 plus 40 kDa MBP tag). Western blotting showed that AMDV-positive sera reacted specifically against a purified 64 kDa VP2332-452 fusion protein (Fig. 1B). No any protein belong to pMAL-c2x was detected in BL21 E. coli.

Thirty mink were immunized with purified inactivated wild-type Chinese AMDV strain Z in complete Freund's adjuvant and boosted twice with incomplete Freund's adjuvant at 2-week intervals (Approved by the Harbin Veterinary Research Institute Animal Center). Sera were collected at 15th day after the final boost; Thirty immunized (n=30) and eight non-immunized mink sera (n=8) were used as positive and negative control samples in ELISA, WB, and CIEP (21, 22). Sera against other known mink pathogens: mink viral enteritis virus, Canine distemper virus, and Aujeszky Disease (Pseudorabies) virus were collected at the Harbin Veterinary Research Institute. 357 clinical serum samples were collected from minks suffering from AMD at various commercial farms since 2011.

CIEP was performed using Danad antigen (Kopenhagen Fur Breeders’ Association, Glostrup, Denmark) by following the manufacturer’s instructions (21, 22). Briefly, Danad antigen was added in opposing wells spaced 1 cm, and the gel run for 30 min in Gelmann buffer (50.5 mM barbiturate, 40 mM Tris) at 4.5 V. A. Precipitate formation in the gel between the two wells indicated positivity.

For ELISA procedures, 96-well ELISA plate (BIOFIL; Canada JET Biochemicals Inc.), 100 μl of sample volumes, and PBS containing 0.05% Tween 20 and 5% skim milk as a dilution buffer were used. To standardize the VP2332-452-ELISA, the AMDV-positive and -negative sera were diluted from 1:50 to 1:6400, and the conjugate from 1:500 to 1:4000. To determine the optimal concentration of protein, a checkerboard titration was carried out with different amounts of VP2332-452 protein (ranging from 7000 to 0.7 ng/ml). By using the AMDV-positive or -negative sera, we found the optimal dilution of the test sera to be 1:100. The optimum VP2332-452 protein concentration was found to be 700 ng/ml. The optimal conjugate dilution was determined to be 1:2000. Thus, standardized VP2332-452-ELISA procedure is 100 μl of 700 ng/ml purified VP2332-452 protein coated onto the wells of a 96-well ELISA plate and incubated overnight at 4°C. After washing twice with PBS-T (PBS containing 0.05% Tween 20), 1:100 diluted
116 AMDV-positive and -negative sera were added. 100 μl of the 1:2000 diluted conjugate was
117 added after washing. Reactions were stopped by adding 3 M NaOH, and the plate was read on a
118 microplate reader (Bio-Rad, Japan) at 405 nm.

119 Using this standardized procedure, a good positive/negative (P/N) ratio was obtained by
120 dividing the positive and negative optical density (OD) values (Fig. 2.1). AMDV-negative (n=8) and
121 -positive sera (n=30) were examined for the presence of specific antibodies in both the
122 VP2332-452-ELISA and CIEP tests. The average OD of the AMDV-negative sera in the
123 VP2332-452-ELISA were 0.30±0.024 (standard deviations [SD]). Cut-off value of the test was
124 determined as 0.372 calculating the arithmetic mean plus three standard deviation of OD values of
125 negative samples. The 1:100-diluted serum specimens with OD of <0.372 or ≥0.372 were
126 interpreted as negative or positive, respectively. According to this criterion, eight uninfected mink
127 sera were negative and thirty AMDV-positive sera were positive in the VP2332-452-ELISA.

128 The detection threshold of the VP2332-452-ELISA compared to that of the CIEP test was
129 determined by using serial dilutions of the AMDV-positive sera. The sensitivity was a dilution of
130 1:3200 sera tested at 0.372 absorbance units for the VP2332-452-ELISA. The negative-control sera
131 showed no detectable VP2332-452-specific antibodies in ELISA. Antisera specific for other known
132 mink pathogens yielded <0.372 OD values, indicating that there was no cross-reactivity between
133 the antisera specific for other known mink pathogens and VP2332-452 protein in ELISA.

134 The sensitivity and specificity of the VP2332-452-ELISA were compared to those of the CIEP
135 test by using the 357 clinical serum samples (Table 1). The CIEP test determined that 261 and 96
136 samples were AMDV-positive and -negative, respectively. The VP2332-452-ELISA result showed
137 that 256 and 101 serum samples were VP2332-452-antibody positive and negative, respectively.
138 Together, 357 serum samples were judged to be 254 positive and 96 negative by both methods
139 (Table 1). Using the CIEP test as a reference, the specificity and sensitivity of the
140 VP2332-452-ELISA were calculated to be 97.9% and 97.3%, respectively. The concordance between
141 the two methods was 98.0%.

142 The western blotting procedure was also optimized for AMDV antibody detection, and the
143 results were compared with those of CIEP. Briefly, Approximately 1 μg purified VP2332-452
144 proteins were subjected to 10% SDS-PAGE and transferred to nitrocellulose membrane. The
membrane was incubated with AMDV-positive (n=30) or -negative (n=8) sera (diluted 1:100 in PBS) followed by a secondary HRP-conjugated goat anti-feline antibody (Supplemental material Figure S1). AMDV-positive sera (n=30) reacted specifically against a purified 64-kDa VP2332-452 fusion protein. No proteins were detected from AMDV-negative (n=8) sera. To compare with the CIEP test, 350 clinical serum samples (that were in agreement for both the CIEP test and the VP2332-452-ELISA) were analyzed by western blotting. Briefly, 1 μg/ml VP2332-452 fusion proteins transferred from polyacrylamid gel on nitrocellulose membrane was probed with 1:100 diluted 350 clinical sera as described for western blotting. The sensitivity and specificity of western blotting were analyzed using the results of the CIEP as a reference test. The specificity and sensitivity were found to be 86.4% and 91.7%, respectively. The results of the CIEP test and the western blotting assays were in agreement for 316 samples (Table 2). However, 21 samples which were found negative in western blotting were positive in CIEP, and 13 sera negative CIEP were positive in the western blotting. The sensitivity and specificity of western blotting were lesser than the VP2332-452-ELISA.

The aims of this study was to develop a cheap, simple, and sensitive methods to detect antibodies against AMDV in mink using VP2332-452 protein, a core antigen of VP2 protein. VP2 protein peak antigen region was calculated, cloned, and expressed in an E. coli system. By using the purified VP2332-452 protein as detecting antigen, clinical mink sera were tested by VP2332-452-ELISA and western blotting. The results of VP2332-452-ELISA and western blotting using the purified recombinant VP2332-452 protein were compared with the results of CIEP using a commercial antigen. The results showed that the recombinant protein VP2332-452 has a good antigenicity to detect AMDV-specific antibodies in ELISA and western blotting. Also, compared to western blotting, the ELISA results were in better concordance with CIEP.

A preferred diagnostic technique requires simple steps, cost-effective, and automated system for intensive surveillance and routine diagnosis of a disease. The VP2332-452-ELISA is such a technique to fulfill such objectives and serves as an inexpensive source of reagents for clinical surveillance of AMDV infection.

In conclusion, the VP2332-452-ELISA has advantages over the CIEP and western blotting, and it can be used for the detection of antibodies against AMDV in mink.

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FIG 1 (A) Identification of VP2_{332-452} fusion protein from transformed *E. coli* cells by SDS-PAGE. Lane 1, *E. coli* transformed with pMAL-VP2_{332-452}; lane 2, *E. coli* transformed with pMAL-c2x; lane M, molecular mass marker. (B) Purified VP2_{332-452} fusion protein analyzed by SDS-PAGE and detected by western blotting with mink anti-AMDV sera. Lane M, molecular mass marker; lane 1, purified VP2332-452 fusion protein; lane 2, protein from pMAL-c2x transformed *E. coli*. 
### TABLE 1 Comparison between CIEP and VP2332-452-ELISA tests for detection of AMDV-related antibodies

<table>
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<th>Test result</th>
<th>CIEP positive</th>
<th>CIEP negative</th>
<th>Total</th>
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<tbody>
<tr>
<td>VP232-452-ELISA positive</td>
<td>254</td>
<td>2</td>
<td>256</td>
</tr>
<tr>
<td>VP232-452-ELISA negative</td>
<td>7</td>
<td>94</td>
<td>101</td>
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<tr>
<td>Total</td>
<td>261</td>
<td>96</td>
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### TABLE 2 Comparison between CIEP tests and western blotting in the detection of ADMV-related antibodies

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<th>CIEP negative</th>
<th>Total</th>
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<tr>
<td>Western blotting positive</td>
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<td>13</td>
<td>246</td>
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<tr>
<td>Western blotting negative</td>
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<tr>
<td>Total</td>
<td>254</td>
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