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Enzyme-Linked Immunosorbent Assay Based on a Chimeric Antigen Bearing Antigenic Regions of Structural Proteins E^{rns} and E2 for Serodiagnosis of Classical Swine Fever Virus Infection

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The antigenic region (residues 109 to 160) of classical swine fever virus (CSFV) protein E^{rns} and the N-terminal antigenic region (residues 1 to 136) of protein E2 were constructed in the form of a fused, chimeric protein, C21E^{rns}E2, for use as an enzyme-linked immunosorbent assay (ELISA) antigen for the serodiagnosis of CSFV infection. Tested with 238 negative-field (CSFV-free) sera from Canadian sources, the specificity of the ELISA was determined to be 93.7%. All 20 sera from experimentally infected pigs representing a variety of animals, virus strains, and days postinfection (dpi; range, 7 to 210) were detected as positive (100%). In contrast, an ELISA based on an E^{rns} fragment ($E^{rns}_{aa\ 109–160}$) or an E2 fragment ($E2_{aa\ 1–221}$) identified only 18 (90%) of 20 sera from infected pigs as positive, missing two targets collected at 7 dpi. These data suggest that use of the chimeric antigen C21E^{rns}E2 would improve serodiagnostic sensitivity and allow for the detection of CSFV infection as early as 7 dpi.

Classical swine fever (CSF) is a highly contagious disease of pigs caused by infection with CSF virus (CSFV), a single positive-stranded RNA virus (26). The CSFV genome (approximately 12.5 kb) contains a single large open reading frame coding for a polyprotein of approximately 4,000 amino acids (aa). The precursor polyprotein is cleaved co- and posttranslationally by cellular and viral proteases into structural proteins C, E^{rns}, E1, and E2 and nonstructural proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B (32). Pigs infected with CSFV can develop various forms of illness: acute, subacute, subclinical, chronic, and late onset (8, 34). The control and eradication of the disease in domestic pigs largely rely on the early diagnosis of infection and/or prevention of infection through vaccination. Laboratory diagnosis of CSFV infection can be achieved by a range of assays based on two principles: (i) the detection of virus itself or viral materials such as antigens and genomic RNA and (ii) the detection of antibodies directed against CSFV. The gold standard for diagnosing CSFV-infected pigs is virus isolation by culture techniques from tissue, whole blood, or blood components using porcine kidney cell lines such as PK15 cells (16, 10). Although the in vitro culture method of virus isolation is sensitive, it is time-consuming (2 to 4 days) and labor-intensive and requires extensive laboratory facilities. Detection of viral antigens can be achieved through examination of the tonsil by immunohistochemical techniques such as the direct immunofluorescence antibody test and the immunoperoxidase test on cryostat-frozen tissue sections (13, 31). Several enzyme-linked immunosorbent assays (ELISAs) have been described for the detection of CSFV antigens in blood or tissue samples (6, 9, 16). The viral genomic RNA can be detected by reverse transcriptase PCR (14, 15, 35), offering better results than other established assays such as virus isolation and antigen capture ELISA (10). The reverse transcriptase PCR procedure is generally considered to be the most sensitive in vitro method for the detection of CSFV-infected pigs and is particularly suitable for the early detection of CSFV infection (10, 13). However, the process of preparing samples is tedious and laborious, making this test less suitable for testing large sample volumes. Antibodies can be detected by either the virus neutralization test or the antibody ELISA (8). The neutralization peroxidase-linked assay (33) and the immunoperoxidase monolayer assay (28) are the two most often used virus neutralization tests. These tests are reliable and sensitive but require cell cultures and are therefore time-consuming. In contrast, the antibody ELISA, mainly using the structural protein E2 (4, 5, 7, 29, 30) or E^{rns} (27) as an antigen, is relatively rapid and suitable for large-scale screening of serum samples, making it a useful diagnostic tool in a CSFV surveillance and eradication program or in a CSFV outbreak situation. Some studies have suggested that antibody detection techniques are of little value for the early detection of CSFV infection (10, 17, 18). Thus, an improved antibody ELISA is still needed to detect all CSFV infections at all possible stages of the immune response. In 2001, Langedijk et al. observed that some individual sera of CSFV-infected animals react differently in the E2 and E^{rns} ELISAs (19). Some sera reacted in the E^{rns} ELISA or E^{rns} peptide ELISA but not in the E2 ELISA and vice versa (19). Thus, a chimeric protein carrying the antigenic regions of E^{rns} and E2 may be utilized to detect more antibody-positive sera than each individual protein can detect.

Recently, we have mapped the antigenic domains of CSFV strain Alfort/187 E2 and E^{rns} using various N- and C-terminal deletion constructs (22, 24). The E2 protein possesses an immunogenic domain located in the N-terminal region of about 120 residues. E^{rns} contains an immunodominant region encompassing three overlapping antigenic regions that induce

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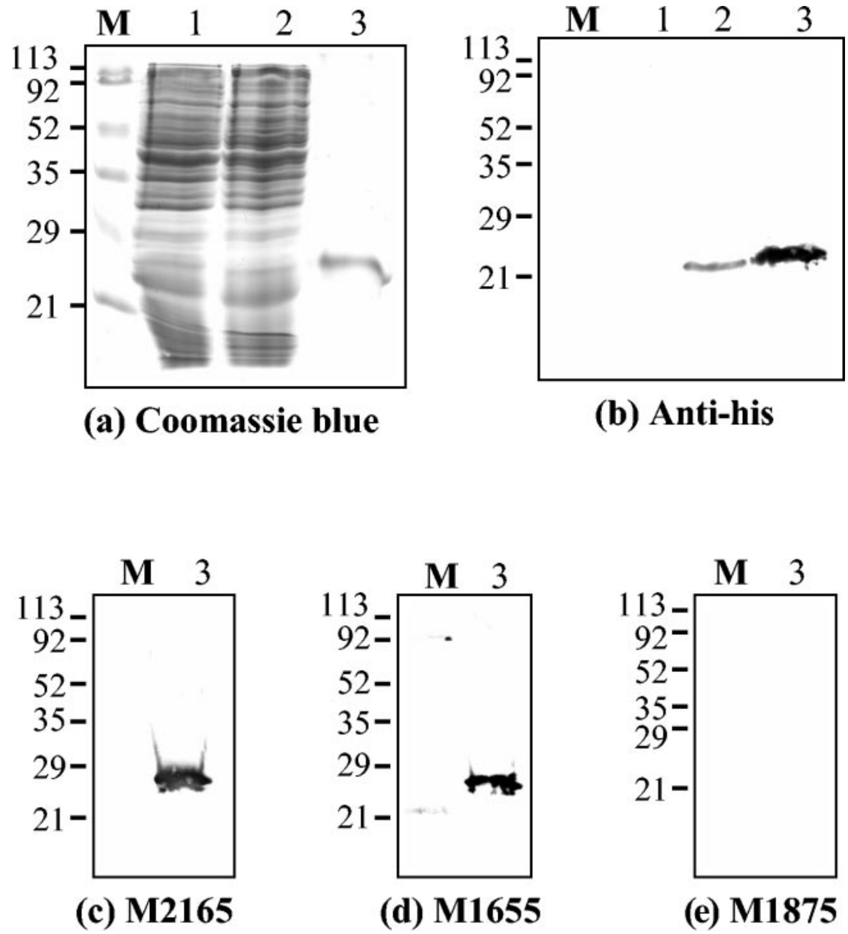

FIG. 1. SDS-PAGE and Western blot analysis of the C21E^{rns}E2 chimeric protein expressed from pET184-177. The protein bands were stained with Coomassie blue (a) or immunostained with anti-His MAb (b), anti-E^{rns} M2165 (c), anti-E2 M1655 (d), or nonspecific MAb M1875 (e). Lanes: M, the protein standards with their molecular masses in kilodaltons shown on the left; 1, whole-cell lysates of an *E. coli* clone (equivalent to 1 ml of culture with an A_{590} of 0.2) harboring pET184-177 in the absence of IPTG (isopropyl-β-D-thiogalactopyranoside); 2, whole-cell lysates of an *E. coli* clone harboring pET184-177 after induction with IPTG for 3 h; 3, nickel chelate affinity-purified C21E^{rns}E2 (5 μg in panel a, 1 μg in all other panels).

antibody responses during CSFV infection: $E^{rns}_{aa\ 65–145}$ (antigenic region 1 [AR1]), $E^{rns}_{aa\ 84–160}$ (AR2), and $E^{rns}_{aa\ 109–220}$ (AR3) (24). The consensus sequence (aa 109 to 145) of the three E^{rns} antigenic regions was found to contain conformational epitopes (23). Elucidation of the antigenic architectures of E2 and E^{rns} has provided a basis for further construction of an immunogenic chimeric protein, C21E^{rns}E2, that fuses an $E^{rns}_{aa\ 109–160}$ fragment (15 aa larger than the consensus region) with the N-terminal 136 residues of E2 as a diagnostic reagent, as described in this study.

The construct pET184-177, coding for C21E^{rns}E2 with an additional fusion of eight residues, including a six-histidine tag at the C-terminal end, was generated using a two-step PCR strategy (21) from the plasmid templates pCR68-69 (24) and pETE2AB (22) under amplification conditions described previously (24). The E^{rns}-specific primers used were P184, 5′-ACACATATGGAGTGCGCTGTGACTTGTAG-3′ (NdeI site underlined), and P181, 5′-AGGCTAGCTGGGAAACATTGAAATTACATG-3′ (a stretch overlapping the E2 gene underlined). The E2-specific primers were P180, 5′-CAATGTTTCCCAGCTAGCCTGCAAGGAAGA-3′ (a stretch overlapping the E^{rns} gene underlined), and P177, 5′-GTGCTCGAGAACACCCGTCCACCCTATTG-3′ (XhoI site underlined). The presence of a correct insert in the construct was verified by DNA sequencing with T7 promoter and T7 terminator primers. The constructs pET167-142 and pETE2AB, coding for $E^{rns}_{aa\ 109–160}$ and the N-terminal 221 amino acids of the CSFV strain Alfort/187 E2 (aa 690 to 910 of the polyprotein, designated E2AB), respectively, were from previous studies (22, 24). The recombinant E2AB ($E2_{aa\ 1–221}$), $E^{rns}_{aa\ 109–160}$, and C21E^{rns}E2 proteins were purified using nickel chelate affinity chromatography as described previously (22, 24), quantified using the Bradford method (2) with bovine serum albumin as a standard, and used as ELISA antigens, respectively.

Expression of the fusion gene encoding the chimeric protein C21E^{rns}E2 in *Escherichia coli* was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blot analysis (24) using an anti-His monoclonal antibody (MAb) (QIAGEN, Santa Clarita, Calif.) probe (Fig. 1a and b). Although the chimeric protein was not evident

TABLE 1. Sera from pigs experimentally infected with various CSFV strains

Serum sample (collection date)[a]	dpi	dpc[b]	CSFV strain
HC hyperimmune serum P15-93 (302-08) (9-15-94)		15	NA[c]
HC hyperimmune serum P16-93 (9-21-94)		21	NA
HC antiserum P43-83 (8-16-83)		17	NA
HC standard serum P78-82 (4-18-83)	166		NA
HC P12-93 (1-12-94)		8	Alfort
HC P17-93 (12-29-93)	56		Glentorf
HC P18-93 (12-29-93)	56		Glentorf
HC P19-93 (12-30-93)	57		Glentorf
HC P4-92 (6-2-92)	7		Glentorf
HC P4-92 (6-30-92)	35		Glentorf
HC P5-92 (6-2-92)	7		Glentorf
HC P5-92 (6-9-92)	14		Glentorf
HC P5-92 (6-16-92)	21		Glentorf
HC P5-92 (6-30-92)	35		Glentorf
HCV hyperimmune serum P222 (6-2-64)	NA		NA
HCV antiserum L-1 P154 (6-2-66)	210		Lapinized
HCV antisera NT P155 (6-22-66)		210	Lapinized
HCV immune sera 51PIC/41 P2427 P3C (1-16-69)	71		NA
HCV antiserum P54-83 (10-24-83)	NA		NA
HC PA03 (12-14-98)	7		Alfort

[a] Collection dates shown are month-day-year. HC, hog cholera; HCV, hog cholera virus.
[b] dpc, days postchallenge. These animals were infected with one CSFV strain and then challenged with other CSFV strains during the course of infection.
[c] NA, not available.

in the SDS-PAGE analysis of total cellular proteins from induced cells (Fig. 1a), a protein band migrating to a position slightly greater than the predicted size (21,393 kDa) of C21E^{rns}E2 was detected by Western blot analysis with an anti-His MAb probe (Fig. 1b). Differences between the apparent and deduced molecular masses of proteins have been generally recognized in a previous study (20). The expressed protein was recognized by both anti-E^{rns} MAb M2165 (Fig. 1c) and anti-E2 MAb M1655 (Fig. 1d) but not by the nonspecific MAb M1875 (Fig. 1e), indicating that it contains the components of E^{rns} and E2 with their antigenic determinants preserved. M2165, specific for the CSFV E^{rns} protein, and M1655, directed against the CSFV E2 protein, were developed in our laboratory (M. Lin, unpublished data). M1875, the MAb to the bluetongue virus core protein VP7, was described previously (25). The chimeric C21E^{rns}E2 protein was purified to near homogeneity with a yield of approximately 2.8 mg protein from 1-liter culture by nickel chelate affinity chromatography (Fig. 1a).

The use of chimeric proteins as serodiagnostic reagents has been described for other viruses (1, 3, 11, 12). To evaluate the chimeric C21E^{rns}E2 protein as a new antigen for the serological detection of CSFV infection, ELISA with the coating antigen at 2 μg/ml was performed essentially as described previously (23) to detect antibodies in 20 sera from 16 experimentally infected pigs representing a variety of animals, virus strains, and days postinfection (dpi) (Table 1). For comparison, the same sera were tested by ELISA using E2AB and E$^{rns}_{aa\ 109–160}$ as antigens. A total of 238 negative serum samples from Canadian sources, collected for a swine survey, were also analyzed in parallel to establish a cutoff that separates positive antibody reactions from negative ones. Receiver operating characteristic (ROC) analysis of the ELISA results was

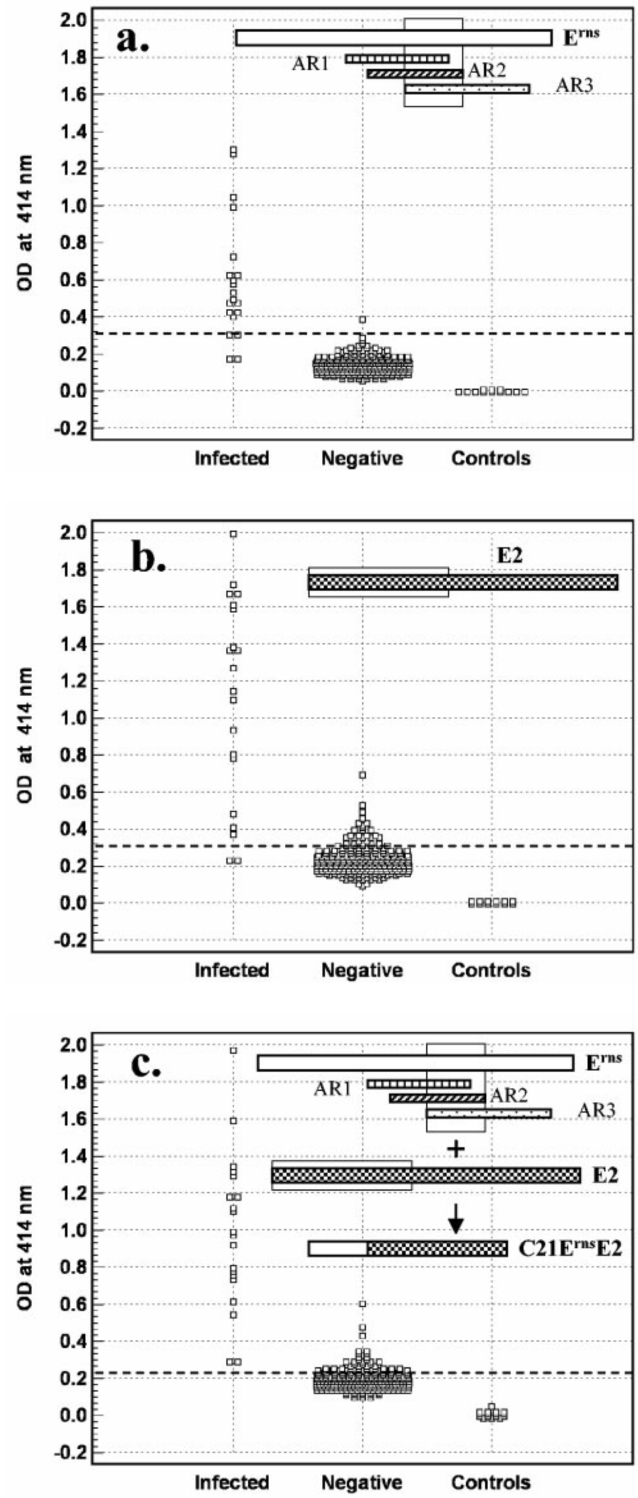

FIG. 2. Detection by ELISA of antibodies in sera of pigs experimentally infected with CSFV. Twenty serum samples from CSFV-infected pigs (Table 1) and 238 CSFV-negative pig serum samples were analyzed for reactivity with immobilized ELISA antigens E$^{rns}_{aa\ 109–160}$ (a), E2AB (b), and C21E^{rns}E2 (c) at 2 μg/ml each. Dilution buffer without testing serum served as a control. The optimum cutoff determined by ROC analysis is indicated by a broken line. The locations of the E^{rns} and E2 fragments relative to the full-length proteins are boxed in the insets. The three overlapping antigenic regions of E^{rns} identified previously (24) are marked as AR1, AR2, and AR3.

performed using the statistical program MedCalc version 7.3 (MedCalc software; Mariakerke, Belgium) as described previously (23). An optimum optical density cutoff at 414 nm, corresponding with the highest accuracy (i.e., minimal false-negative and false-positive results), was determined to be 0.228 by ROC analysis for the chimeric protein (Fig. 2c). Based on the cutoff, the serological specificity was 93.7%; 20 (100%) out of 20 serum samples from infected pigs were detected as positive for the presence of anti-CSFV antibodies. The ROC analysis yielded an area under the curve of 0.993, with a 95% confidence interval (CI) of 0.974 to 0.999. Similarly, the ROC analysis derived optimum cutoffs of 0.369 and 0.287 for the $E^{rns}{}_{aa\ 109–160}$ and E2AB ELISAs, respectively (Fig. 2a and b). Based on these cutoffs, the serological specificities were 99.6% and 94.5% for $E^{rns}{}_{aa\ 109–160}$ and E2AB, respectively. Both detected 18 (90%) out of 20 serum samples from infected pigs as positive reactors. Two serum samples collected at 7 dpi from infected pigs were not picked up by the $E^{rns}{}_{aa\ 109–160}$ or E2AB ELISA alone. The ROC analysis yielded areas under the curve of 0.979 (95% CI, 0.953 to 0.993) for $E^{rns}{}_{aa\ 109–160}$ and 0.947 (95% CI, 0.912 to 0.971) for E2AB. In practice, a cutoff greater or less than the optimum may be chosen to achieve the desired specificity or sensitivity for an assay. Collectively, the ELISA results indicated that incorporation of the C21E^{rns}E2 chimera into the assay test has improved the serodiagnosis of CSFV infection over that with individual E^{rns} or E2 fragments, i.e., $E^{rns}{}_{aa\ 109–160}$ or E2AB. Importantly, the C21E^{rns}E2 chimera can detect CSFV infection as early as 7 dpi. In contrast, the entire E^{rns} protein cannot detect CSFV-specific antibodies in vaccinated or unvaccinated pigs earlier than 14 dpi (27). In addition, an E2-based competitive ELISA can detect 97% of neutralization peroxidase-linked assay-positive pig sera at 21 dpi or later (5). It appears that the chimera combining the immunogenic regions of E^{rns} and E2 in a single polypeptide offers an advantage in its ability to detect CSFV infection in the early stages. It has been observed that some individual sera of CSFV-infected animals react differently in the E2 and E^{rns} ELISAs (19). Some sera react in the E^{rns} ELISA or E^{rns} peptide ELISA but not in the E2 ELISA and vice versa (19). This observation suggests that the use of a chimeric antigen such as C21E^{rns}E2 that combines the antigenic regions of both E^{rns} and E2 in a single polypeptide would improve diagnostic sensitivity. This is supported by the current finding that a C21E^{rns}E2-based ELISA is capable of detecting the presence of CSFV-specific antibodies in two serum samples collected at 7 dpi, which were not detected by either the E^{rns} or the E2 fragment alone.

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