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Antibody Responses of Pigs to Defined E^{rns} Fragments after Infection with Classical Swine Fever Virus

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Antibody responses of pigs to defined E^{rns} fragments, after classical swine fever virus (CSFV) infection, were studied by using an enzyme-linked immunosorbent assay (ELISA). Selection of various E^{rns} fragments was based on an immunodominant E^{rns} region encompassing three overlapping antigenic regions, amino acids 65 to 145 ($E^{rns}_{aa\ 65–145}$) (AR1), 84 to 160 ($E^{rns}_{aa\ 84-160}$) (AR2), and 109 to 220 ($E^{rns}_{aa\ 109-220}$) (AR3), identified earlier by our group (M. Lin, E. Trottier, J. Pasick, and M. Sabara, J. Biochem., in press). Defined E^{rns} fragments, including AR1, AR2, AR3, $E^{rns}_{aa\ 65-160}$ (AR12), $E^{rns}_{aa\ 84-220}$ (AR23), $E^{rns}_{aa\ 65-220}$ (AR123), $E^{rns}_{aa\ 109-145}$ (the consensus region defined by the three overlapping regions), and $E^{rns}_{aa\ 109-160}$ (a fragment 15 amino acids larger than the consensus region), were expressed in *Escherichia coli*, purified by nickel chelate affinity chromatography, and used to measure antibody responses in 20 sera serially collected from pigs experimentally infected with CSFV. Based on the optimum cutoffs determined by receiver operating characteristic analysis after testing 238 negative field sera from Canadian sources, all the E^{rns} fragments were capable of distinguishing positive from negative antibody responses with sensitivities ranging between 75 and 90% and specificities ranging between 83.2 and 100%. Detection of antibody responses to refolded $E^{rns}_{aa\ 109-145}$ and $E^{rns}_{aa\ 109-160}$ by ELISA (this study) but not by Western blots (Lin et al., in press) indicated that the epitopes within the consensus region are conformational. When cutoff values were raised to give a specificity of 100%, four E^{rns} fragments (AR2, AR23, $E^{rns}_{aa\ 109-145}$, and $E^{rns}_{aa\ 109-160}$) offered much higher sensitivities (75 to 90%) than those obtained with other fragments (20 to 65%). $E^{rns}_{aa\ 109-145}$ and $E^{rns}_{aa\ 109-160}$ were capable of detecting antibody responses in infected pigs as early as 7 days postinfection. Demonstration of antibody responses to either one of the four fragments can thus be an alternative to use of the full-length protein in ELISA for serological diagnosis of CSFV infection. An advantage of such a test would be its utilization for serological survey in a classical swine fever-free country (e.g., Canada) in biocontainment level 2 laboratories.

Classical swine fever virus (CSFV) is an enveloped positive-stranded RNA virus (20) of the genus *Pestivirus* of the *Flaviviridae* family (34). Other viruses classified in this genus are *Bovine viral diarrhea virus* and *Border disease virus* of sheep. CSFV is highly contagious and can cause a fatal disease in pigs. The disease, characterized by fever and hemorrhage, can be acute, chronic, or subclinical with substantial economic loss. The classical swine fever outbreak in The Netherlands during 1997 and 1998 resulted in destruction of more than 10 million pigs at a cost of more than 2 billion U.S. dollars (25). Similar to other members of the genus, the 12.5-kb CSFV genome contains a single large open reading frame encoding a polyprotein precursor of approximately 4,000 amino acids (aa). The precursor 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 (26).

The biochemical, biological, and functional properties of the envelope glycoprotein E^{rns} have been studied in some detail. This protein forms a disulfide-bridged homodimer in the virion (31, 15) and is highly modified by N-linked glycosylation, which contributes about 50% to the molecular mass of E^{rns} (28, 36). The glycoprotein is both virion associated and secreted, since it is found both on the surface of pestivirus-infected cells and in the culture medium (28). E^{rns} does not contain a potential membrane-spanning domain, yet its C-terminal domain can translocate the full-length protein across eukaryotic cell membranes (13). The protein can bind to several cell types and inhibits CSFV and bovine viral diarrhea virus infection in cell culture, suggesting that E^{rns} is involved in attachment to or entry of the viruses into susceptible cells (7). This interaction is believed to be mediated with cell surface glycoaminoglycans, such as heparan sulfate (9, 10). The E^{rns} protein contains RNase activity (5, 6, 30, 36), which is a unique feature for a viral surface protein. Homology in two stretches of the E^{rns} sequence to members of the Rh/T2/S RNase superfamily provides a structural basis for the RNase activity (15, 30). The biological function of E^{rns} RNase activity is not fully understood; however, destruction of the RNase activity by mutations gives rise to viruses that are more cytopathic in culture and attenuated in vivo (8, 19). Antibodies that inhibit RNase activity also tend to neutralize CSFV infectivity (36). Although cytotoxicity is a feature of other soluble ribonucleases (29), the link has yet to be established for E^{rns}.

Antibodies directed against E^{rns}, E2, and NS3 have been demonstrated in infected animals (14, 16, 21, 22, 24), with E^{rns} and E2 capable of inducing neutralizing antibodies (11, 12, 32). It has been shown that antibodies to full-length E^{rns} or even a 37-mer peptide derived from its C-terminal end (aa 191 to 227)

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

Serum	Time of testing[a]	CSFV strain used
S#1 HC HyperImmune Serum P15-93 (302-08) 94-09-15	15 dpc 4	NA
S#2 HC HyperImmune Serum P16-93 94-09-21	21 dpc 4	NA
S#3 HC Antiserum P43-83 83-08-16	17 dpc 5	NA
S#4 HC Standard Serum P78-82 83-04-18	166 dpi	NA
S#5 HC P12-93 94-01-12	8 dpc	Alfort
S#6 HC P17-93 93-12-29	56 dpi	Glentorf
S#7 HC P18-93 93-12-29	56 dpi	Glentorf
S#8 HC P19-93 93-12-30	57 dpi	Glentorf
S#9 HC P4-92 92-06-02	7 dpi	Glentorf
S#10 HC P4-92 92-06-30	35 dpi	Glentorf
S#11 HC P5-92 92-06-02	7 dpi	Glentorf
S#12 HC P5-92 92-06-09	14 dpi	Glentorf
S#13 HC P5-92 92-06-16	21 dpi	Glentorf
S#14 HC P5-92 92-06-30	35 dpi	Glentorf
S#15 HCV HyperImmune Serum P222 64-06-02	NA	NA
S#16 HCV Antiserum L-1 P154 66-06-02	210 dpi	Lapinized
S#17 HCV Antisera NT P155 66-06-22	210 dpc	Lapinized
S#18 HCV Immune Sera 51PIC/41 P2427 P3C 69-01-16	71 dpi	NA
S#19 HCV Antiserum P54-83 83-10-24	NA[b]	NA
S#20 HC PA03 98-12-14	7 dpi	Alfort

[a] Animals tested *n* dpc were infected with one CSFV strain followed by challenge with other CSFV strains during the course of infection.
[b] NA, not available.

could be used as an indicator of CSFV infection in pigs (14, 21). Recent studies have indicated that an E^{rns}-based enzyme-linked immunosorbent assay (ELISA) can be used as a companion diagnostic test to identify CSFV-infected pigs in herds vaccinated with the E2 subunit marker vaccine (4, 21). However, very little is known about the structural immunogenic organization of E^{rns}. This information would be invaluable for the development of a serological diagnostic test with high sensitivity and specificity. Recently, we have mapped an immunodominant region encompassing three overlapping antigenic regions (ARs) that induce antibody responses during CSFV infection: aa 65 to 145 ($E^{rns}_{aa\ 65–145}$) (AR1), aa 84 to 160 ($E^{rns}_{aa\ 84–160}$) (AR2), and 109 to 220 ($E^{rns}_{aa\ 109–220}$) (AR3) (17). Assignment of these antigenic regions correlates well with the three-dimensional structural model of E^{rns} derived from disulfide bond connectivity and homology modeling (15). Interestingly, the consensus region of the three E^{rns} antigenic regions contained one complete and one partial T-cell epitope sequence as described previously (1). In this study, we measured and compared the antibody responses of pigs to AR1, AR2, AR3, combinations of two or three individual overlapping regions, and the consensus region after experimental infection. The data reported in this study support the selection of an E^{rns} fragment for early detection of antibody in CSFV-infected pigs.

MATERIALS AND METHODS

Protein expression clones. Protein expression constructs pET217-143, pET133-142, pET167-218, pET217-142, pET133-218, pET217-218, pET167-143, pET167-142, and pET134-131 were generated (17) to code for $E^{rns}_{aa\ 65–145}$ (AR1), $E^{rns}_{aa\ 84–160}$ (AR2), $E^{rns}_{aa\ 109–220}$ (AR3), $E^{rns}_{aa\ 65–160}$ (AR12), $E^{rns}_{aa\ 84–220}$ (AR23), $E^{rns}_{aa\ 65–220}$ (AR123), $E^{rns}_{aa\ 109–145}$, $E^{rns}_{aa\ 109–160}$, and $E^{rns}_{aa\ 121–227}E1_{aa\ 1–7}$, respectively. Recombinant proteins expressed from the constructs have an N-terminal fusion of 49 to 58 residues, including a six-histidine tag, and an additional fusion of 8 residues at the C-terminal end.

Sera. Twenty sera from pigs experimentally infected with CSFV (3) were provided by the National Centre for Foreign Animal Diseases (Winnipeg, Manitoba, Canada) and are listed in Table 1. A total of 238 pig serum samples from Canadian herds (classical swine fever [CSF] free) were used for this study.

Expression of recombinant E^{rns} fragments. *Escherichia coli* BL21(DE3)pLysS cells (Novagen, Madison, Wis.) harboring one of the above expression constructs were cultured at 37°C with shaking in 2 to 4 liters of Luria-Bertani broth supplemented with 30 μg of kanamycin/ml until the optical density at 590 nm (OD_{590}) reached between 0.5 and 1.0. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM, and the cells were allowed to express the recombinant proteins for 3 h. The cells were harvested by centrifugation at 10,000 × *g* for 20 min.

Purification of recombinant proteins. Cell pellets were resuspended in phosphate-buffered saline (PBS) (pH 7.2) with 1 mM phenylmethylsulfonyl fluoride and lysed with a French press at 1,500 lb/in^2. The homogenates were spun at 27,000 × *g* for 20 min at 4°C, and the pellet fraction was resuspended in protein extraction buffer (6 M guanidine hydrochloride, 0.1 M NaH_2PO_4, 0.01 M Tris [pH 8.0]). The solubilized sample was spun again at 27,000 × *g* for 20 min at 4°C, and the supernatant was loaded onto a column (1 by 3.5 cm) of nickel-nitrilotriacetic acid agarose (QIAGEN, Santa Clarita, Calif.). The column was washed with buffer A (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris [pH 8.0]), followed by buffer B (buffer A plus 0.5 M NaCl [pH 6.3]) and buffer C (buffer B plus 5 mM imidazole [pH 5.9]). The denatured protein was then refolded on the column by washes with Tris-buffered saline (TBS) (pH 7.4) containing 1 M urea, followed by TBS (pH 7.4). The recombinant protein was eluted and collected with fractions of 1 ml from the column using TBS (pH 7.4) containing 200 mM imidazole. The protein fragment $E^{rns}_{aa\ 109–160}$ was further purified by anion exchange chromatography on a column (1.5 by 4 cm) of Q-Sepharose (Amersham Biosciences, Baie d'Urfe, Quebec, Canada) equilibrated with 50 mM phosphate buffer pH (7.8) and eluted over a 0 to 1 M NaCl linear gradient (60 ml) in phosphate buffer. Proteins were quantified by using the Bradford method (2) with bovine serum albumin as a standard.

Optimization of ELISA conditions. Nunc Maxisorp ELISA plates were coated with 1 or 2 μg of purified antigen/ml to determine the optimal concentration of antigen. The purified protein in 0.06 M carbonate buffer (sodium bicarbonate and sodium carbonate [pH 9.6]) was aliquoted into each well (100 μl) and allowed to incubate at room temperature overnight (18 to 20 h). The plates were then frozen at −20°C and were thawed before use. Plates were washed with PBS (pH 7.2) containing 0.05% Tween 20 (PBST). A positive CSFV-infected pig serum (17 days postinfection [dpi]) and a CSFV-negative pig serum were diluted 1/50 and 1/100 in PBST; 100 μl was applied to each well and incubated for 1 h. The plates were washed and incubated for 1 h with horseradish peroxidase-rabbit anti-swine immunoglobulin G (whole molecule) antibody (1/2,000 dilution in PBST), 100 μl/well. The plates were washed and developed for 10 min with shaking in a solution (100 μl/well) of 1 mM 2,2′-azinobis(3-ethylbenzthiazoline-sulfonic acid) and 0.015% H_2O_2 in 50 mM citrate buffer (26 mM trisodium citrate, 24 mM citric acid [pH 4.5]). The absorbances at 414 nm were measured

on a Labsystems Multiskan bichromatic plate photometer. In addition, bovine serum albumin (3%) as a blocking reagent and the presence of the divalent cation chelating agents EDTA and EGTA (7.5 mM each; pH 6.3) during the serum incubation step were examined to determine whether these reagents could improve the assay.

Detection of pig antibodies to E^{rns} fragments by ELISA. Based on optimization results, all ELISAs were performed with the purified antigen coated at 2 μg/ml and the pig sera diluted 1/100 in PBST containing 7.5 mM of each of EDTA and EGTA (pH 6.3) to reduce the background signal. Twenty CSFV antisera from experimentally infected pigs representing a variety of animals, virus strains, and days postinfection (Table 1) were tested with all nine defined E^{rns} fragments. Also, 238 negative pig sera were tested for each protein.

Data analysis. Receiver operating characteristic (ROC) analysis of the ELISA results was performed using the statistical program MedCalc, version 7.3 (MedCalc Software, Mariakerke, Belgium). The values for the area under the ROC curve, the 95% confidence interval (CI) for the area, and cutoff points were derived from the ROC curve analysis. An area of X means that a randomly selected individual from the positive group has a test value larger than that for a randomly chosen individual from the negative group 100X % of the time. A test that gives a perfect separation of the values of the two groups has an area of 1, whereas a noninformative test that cannot distinguish between the two groups has an area of 0.5. A 95% CI for the area, which does not include the 0.5 value, indicates that the test has an ability to distinguish between the two groups.

RESULTS

Selection of assay parameters. Pig sera at 1:50 and 1:100 dilutions were tested for the presence of CSFV-specific antibodies with ELISA at E^{rns} fragment antigen concentrations of 1 or 2 μg/ml (Fig. 1). Several recombinant proteins, $E^{rns}_{aa\ 112–227}E1_{aa\ 1-7}$, $E^{rns}_{aa\ 109–160}$, and $E^{rns}_{aa\ 109–145}$, were used in the initial evaluation. For the positive serum, the antigens at 2 μg/ml consistently showed higher OD_{414} values than those obtained at 1 μg/ml. The negative serum, in contrast, did not demonstrate any measurable difference in reading when the antigen coating concentration was altered. Thus, the coating concentration of a selected antigen was set at 2 μg/ml to maximize the detection of positive antibody response in sera from infected pigs. For the majority of the proteins used in this evaluation step (at 2 μg/ml), the positive serum at either a 1:50 dilution or a 1:100 dilution gave consistent OD_{414} values greater than 1.00. This indicates that positive antibody responses to selected E^{rns} fragments are well detected at a serum dilution of 1:100. One exception was $E^{rns}_{aa\ 112–227}E1_{aa\ 1–7}$, which at a 1:50 serum dilution did not exceed a reading of 0.70 and which at 1:100 dropped to below 0.50. The negative sera gave only slightly lower readings at 1:100 than at 1:50. To further optimize the assay conditions, bovine serum albumin (BSA) (3%) as a blocking reagent and divalent cation chelating agents EDTA and EGTA (7.5 mM each; pH 6.3), which have been shown to reduce nonspecific serum protein interaction in enzyme immunoassay (23), were explored in the assay (Fig. 2). BSA did not significantly alter the OD_{414} readings of either the positive or negative serum. Thus, this blocking reagent was excluded from the ELISA assays. The presence of EDTA and EGTA during the serum incubation step increased the OD_{414} values for the positive sera and slightly decreased the readings for the negative sera (Fig. 2). Based on these results, subsequent ELISA procedures were standardized to use the coating antigen at 2 μg/ml and serum samples at a 1:100 dilution in PBS containing the chelating agents EDTA and EGTA at 7.5 mM each (pH 6.3).

Antibody responses to individual antigenic regions (AR1, AR2, and AR3) of E^{rns}. The reactivity of antibodies from CSFV-infected pigs with E^{rns} fragments AR1, AR2, and AR3 was examined by using ELISA (Fig. 3). Two hundred thirty-eight pig serum samples from Canadian herds (CSF free) were also analyzed in parallel to establish a cutoff that distinguishes positive antibody responses from negative responses. An optimum cutoff at OD_{414}, corresponding with the highest accuracy (i.e., minimal false-negative and false-positive results), of

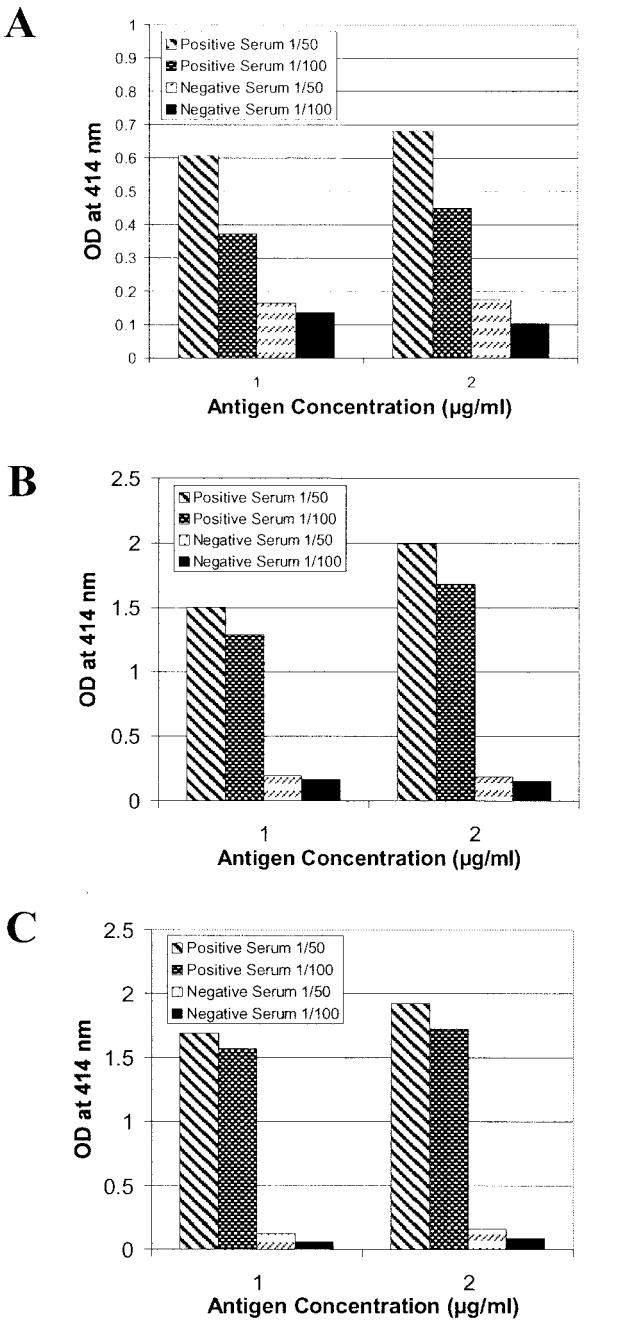

FIG. 1. Optimization of the concentration of coating antigens and dilutions of serum for ELISA. Several recombinant proteins, $E^{rns}_{aa\ 112–227}E1_{aa\ 1–7}$ (A), $E^{rns}_{aa\ 109–160}$ (B), and $E^{rns}_{aa\ 109–145}$ (C), at 1 and 2 μg/ml, were tested for reactivity with one positive serum (S#3) and one negative serum at 1/50 and 1/100 dilutions, respectively. Each bar represents the mean value of two determinations.

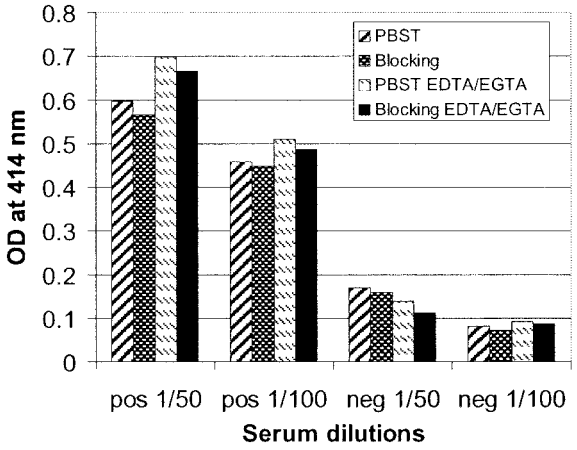

FIG. 2. Influence of BSA (blocking reagent) and divalent cation chelating agents on ELISA. $E^{rns}_{aa\ 112–227}E1_{aa\ 1–7}$ (2 μg/ml) was immobilized on an ELISA plate and reacted with one positive serum (S#3) and one negative serum at 1/50 and 1/100 dilutions. Four assay conditions were examined for each serum dilution: (i) dilution of pig sera in PBST; (ii) blocking of the plate with 3% BSA in PBST prior to adding pig serum (diluted in PBST); (iii) dilution of pig serum in PBST containing EDTA/EGTA (7.5 mM each; pH 6.3); and (iv) blocking of the plate with 3% BSA in PBST prior to adding pig serum (diluted in PBST containing EDTA and EGTA).

0.199, 0.32, and 0.294 was determined by ROC analysis for AR1, AR2, and AR3, respectively. Based on these cutoffs, the serological specificities were 83.2, 98.3 and 92.4% for AR1, AR2, and AR3, respectively; 18 (90%), 17 (85%), and 15 (75%) out of 20 serum samples from 16 infected pigs (Table 1) were identified as having positive antibody responses to these fragments. The ROC analysis yielded an area under the curve (AUC) of 0.898, with a 95% CI from 0.854 to 0.932 for AR1, 0.952 (95% CI = 0.919 to 0.975) for AR2, and 0.841 (95% CI = 0.790 to 0.883) for AR3. These values can be interpreted as described in Materials and Methods. Higher cutoff values that resulted in 100% specificity (no false positives) resulted in detection of 4 (20%), 14 (70%), and 5 (25%) out of 20 infected pig serum samples for AR1, AR2, and AR3, respectively.

Antibody responses to E^{rns} fragments encompassing two to three overlapping individual antigenic regions. Antibody responses in CSFV-infected pigs to the E^{rns} fragments corresponding to overlapping antigenic regions AR12, AR23, and AR123 were evaluated by ELISA using the same serum samples as described above (Fig. 4). The ROC analysis derived an optimum cutoff of 0.361, 0.4, and 0.247 for AR12, AR23, and AR123, respectively. Based on these cutoffs, the serological specificity was 95.8, 100 and 90.8% for AR12, AR23, and AR123, respectively; 16 (80%), 18 (90%), and 18 (90.0%) out of 20 serum samples from infected pigs were identified as having positive antibody responses to these fragments. The ROC analysis yielded an AUC of 0.911 (95% CI = 0.870 to 0.943) for AR12, 0.984 (95% CI = 0.960 to 0.995) for AR23, and 0.910 (95% CI = 0.868 to 0.942) for AR123. Higher cutoff values that resulted in 100% specificity (no false-positive results) resulted in detection of 13 (65%), 18 (90%), and 6 (30.0%) out of 20 serum samples from infected pigs for AR12, AR23, and AR123, respectively.

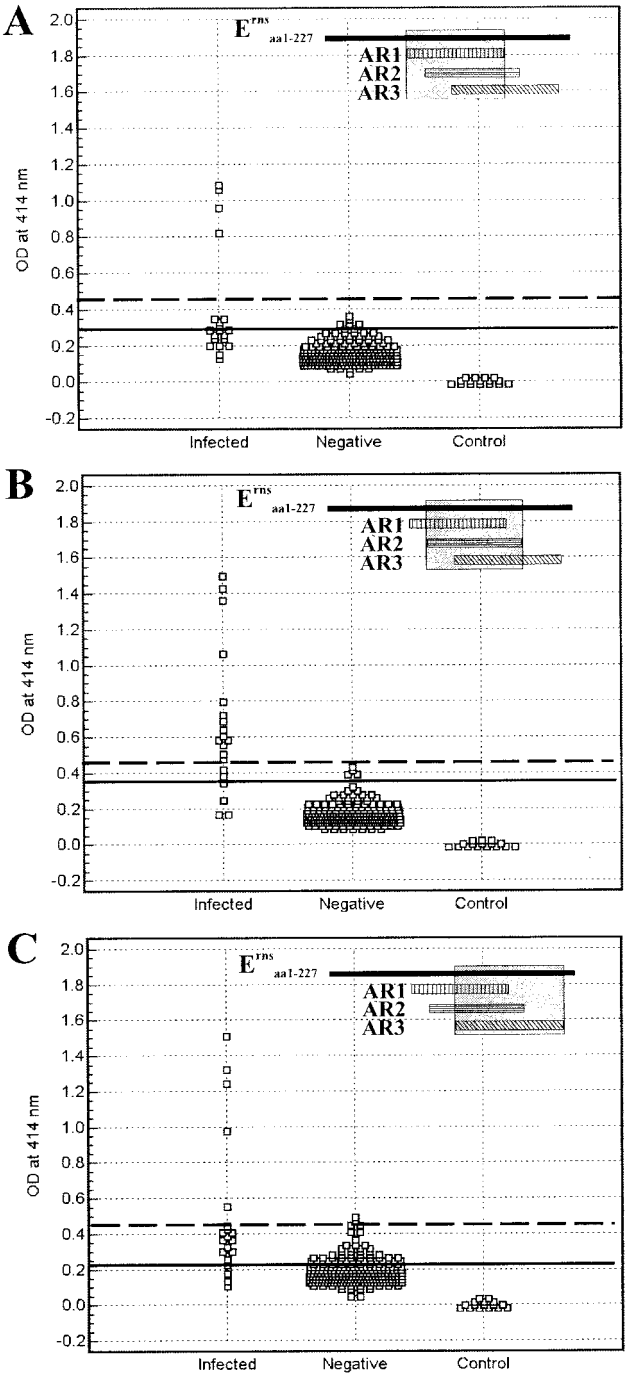

FIG. 3. Detection of antibody responses of pigs to AR1, AR2, and AR3 of E^{rns} by ELISA. Twenty serum samples from CSFV-infected pigs and 238 negative pig sera were analyzed for reactivity with immobilized AR1 (A), AR2 (B), and AR3 (C), using the optimized conditions as described in the text. Dilution buffer without testing serum served as a control. The optimum cutoff determined by ROC analysis is indicated by a solid line. A higher cutoff eliminating all false positives (100% specificity) is indicated by a dashed line. The location of each E^{rns} fragment relative to the full-length protein is marked (shaded) in the insets.

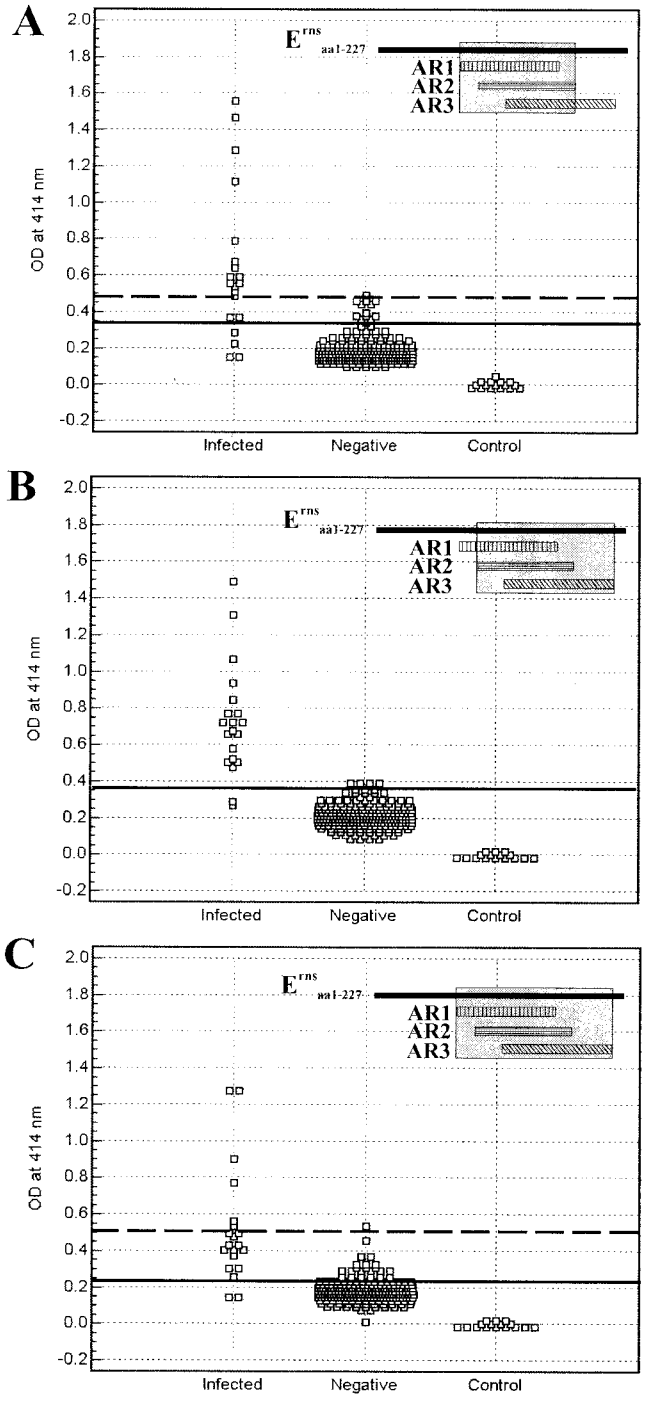

FIG. 4. Detection of antibody responses of pigs to AR12, AR23, and AR123 of E^{rns} by ELISA. E^{rns} fragments corresponding to overlapping antigenic regions AR12 (A), AR23 (B), and AR123 (C) were used as coating antigens. The assay conditions and data presentation are as described in the legend to Fig. 3.

Antibody responses to the consensus region of overlapping AR1, AR2, and AR3. Reactivities of antibodies in sera from CSFV-infected pigs to the consensus region ($E^{rns}_{aa\ 109–145}$) defined by AR1, AR2, and AR3 and $E^{rns}_{aa\ 109–160}$, a fragment that is 15 amino acids larger than the consensus region, are shown in Fig. 5. Optimum cutoff values were determined by ROC analysis to be 0.366 and 0.287 for $E^{rns}_{aa\ 109–145}$ and $E^{rns}_{aa\ 109–160}$, respectively. Based on these cutoffs, the serological specificity was 99.6 and 99.6% for $E^{rns}_{aa\ 109–145}$ and $E^{rns}_{aa\ 109–160}$, respectively; 16 (80%) and 18 (90%) out of 20 serum samples from infected pigs exhibited positive antibody responses to these E^{rns} fragments. The ROC analysis yielded an AUC of 0.937 (95% CI = 0.900 to 0.963) for $E^{rns}_{aa\ 109–145}$ and 0.979 (95% CI = 0.953 to 0.993) for $E^{rns}_{aa\ 109–160}$. Higher cutoff values that resulted in 100% specificity (no false positive) resulted in detection of 15 (75%) and 16 (80%) out of 20 infected pig serum samples for $E^{rns}_{aa\ 109–145}$ and $E^{rns}_{aa\ 109–160}$, respectively.

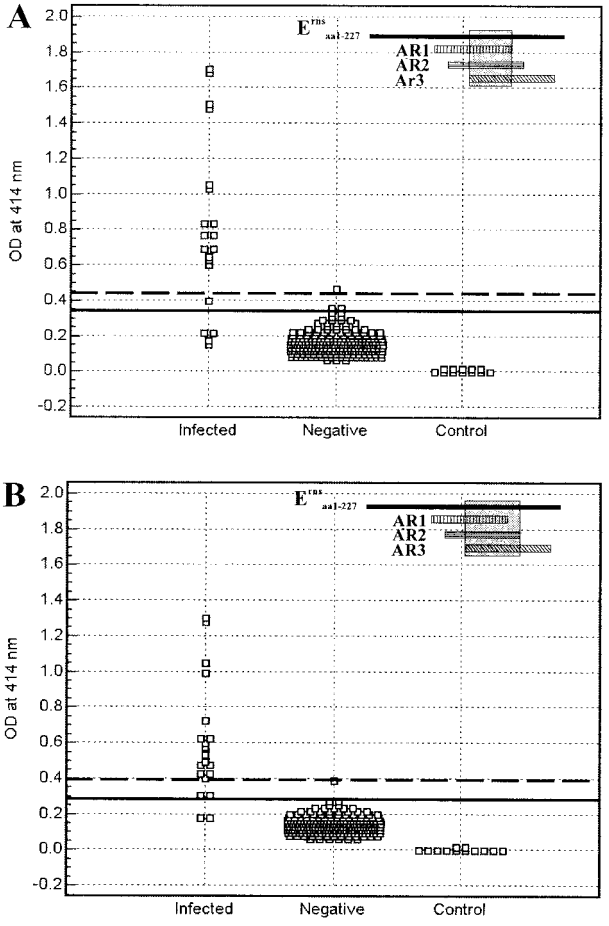

FIG. 5. Detection of antibody responses to the consensus region of overlapping AR1, AR2, and AR3. $E^{rns}_{aa\ 109–145}$ (A), corresponding to the consensus region defined by AR1, AR2, and AR3, and $E^{rns}_{aa\ 109–160}$ (B), a fragment that is 15 amino acids larger than the consensus region, were used as coating antigens. The assay conditions and data presentation are as described in the legend to Fig. 3.

Antibody responses of CSFV-infected pigs to the C-terminal end (37 aa) of E^{rns} were demonstrated earlier by Langedijk et al. (14). Reactivity of an E^{rns} fragment ($E^{rns}_{aa\ 121–227}E1_{aa\ 1–7}$) containing this C-terminal end with sera from CSFV-infected pigs and negative pig sera were also evaluated in the present study. As shown in Fig. 6, the $E^{rns}_{aa\ 121-227}E1_{aa\ 1-7}$ fragment exhibits a weaker reaction with pig antisera than other defined E^{rns} fragments.

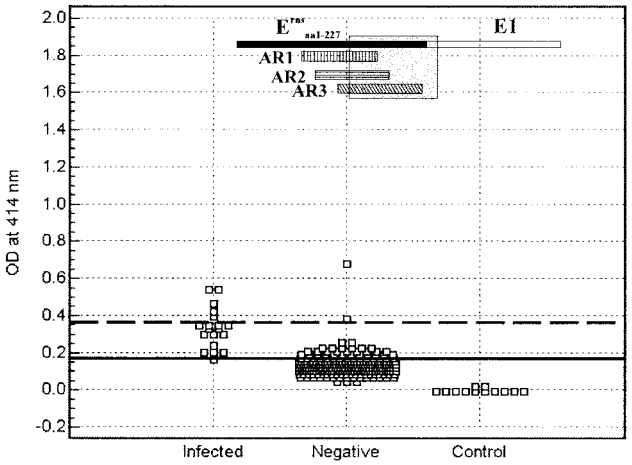

FIG. 6. Detection of antibody responses to the $E^{rns}_{aa\ 121–227}E1_{aa\ 1–7}$ fragment containing the C-terminal end (37 residues). The assay conditions and data presentation are as described in the legend to Fig. 3.

DISCUSSION

Several proteins were previously reported to elicit a humoral immune response in pigs after infection with CSFV (14, 16, 18, 21, 22, 27, 24, 33, 35). Of these immunogenic proteins, E2 and E^{rns} are particularly of importance for the development of diagnostic tests and/or subunit marker vaccine (4). E^{rns}-based ELISA can be a companion diagnostic test for CSFV-infected pigs in an E2-vaccinated herd (4, 21). In this study, we evaluated antibody responses of CSFV-infected pigs to nine defined E^{rns} fragments corresponding to each of the three previously identified overlapping ARs (17), combinations of two to three overlapping ARs, and the consensus region of the three ARs. The study has generated information useful for designing a diagnostic test to improve the serological detection of CSFV infection.

ROC analysis revealed that there is a significant difference in the antibody responses to individual ARs. When the cutoffs were raised above the optimum value calculated by ROC analysis to completely eliminate the false-positive reactions in negative samples, AR1 and AR3 reacted with only a few sera of CSFV-infected pigs, such as S#3, S#7, S#18, S#19, and S#14 (reactive with AR3 only). All of these reactive sera were collected at 35 days postinfection (dpi) or later. This tends to suggest that antibody responses to AR1 or AR3 appear during the late stages of infection. In contrast, AR2 could be used to detect antibodies in a much greater number of sera from infected animals, ranging from 14 to 210 dpi (S#12 and S#16). This indicates that antibody responses directed against AR2 occurred during both early and late infection. Antibody responses to the C-terminal end (37 aa) of E^{rns} were previously demonstrated to be an indicator of CSFV infection (14). For comparison, antibody responses to the C-terminal end (37 residues)-containing fragment $E^{rns}_{a\ a121-227}E1_{aa\ 1–7}$ were also determined (Fig. 6). This fragment, though not recognized by antisera from CSFV-infected pigs on Western blots (17), was able to react with anti-CSFV antisera on ELISA after refolding and eluting from a Ni-agarose column using the method previously described (16). Reaction of $E^{rns}_{aa\ 121–227}E1_{aa\ 1–7}$ with pig antisera was much weaker than that of other defined E^{rns} fragments, indicating that this region is not immunodominant. This further supports the immunodominant nature of an E^{rns} region encompassing three overlapping antigenic regions, AR1, AR2, and AR3 (17).

As expected, the combination of overlapping AR1 and AR2 or AR2 and AR3 (i.e., AR12 or AR23) allowed detection of more positive antibody responses in the 20 serum samples from CSFV-infected pigs than AR1 or AR3 alone at the cutoff of 100% specificity (Fig. 4). Compared to AR2, AR12 resulted in a similar number of positive antibody responses, while use of AR23 detected more positive responses. This indicates that AR2 makes a major contribution to the sensitivity of detection from the overlapping combination; the contribution of AR1 is insignificant, while AR3 appears to enhance the detection sensitivity. Surprisingly, the number of antibody responses to AR123 is similar to that with AR1 or AR3 but is much smaller than that with AR2. The reason for this unexpected result is not clear. Perhaps some antibody binding sites could have been masked by the additional sequences surrounding AR2.

Our previous data showed that $E^{rns}_{aa\ 109–145}$, the consensus region defined by AR1, AR2, and AR3, and $E^{rns}_{aa\ 109–160}$, a fragment 15 amino acids larger than the consensus region, were not recognized by CSFV antisera on Western blots (17). The present data show that both $E^{rns}_{aa\ 109–145}$ and $E^{rns}_{aa\ 109–160}$, after refolding and eluting from a Ni-agarose column, were recognized by antibodies in sera from CSFV-infected animals in ELISA. This result provides evidence that the epitopes located within the consensus region are conformational. This is consistent with the proposed three-dimensional model of E^{rns} (15), which predicts the cysteine-rich consensus region to be on a large surface-exposed loop between helix 6 and helix 7 and likely to form two disulfide bonds in this region. A comparison of the ELISA results from $E^{rns}_{aa\ 109–145}$ and $E^{rns}_{aa\ 109–160}$ with a larger E^{rns} fragment (AR123) that contains the consensus sequences reveals that the consensus region detected more positive serum samples than AR123 at a 100% specificity cutoff. This, together with other results, suggests that extra residues added to the N-terminal end of $E^{rns}_{aa\ 109–145}$ reduce the binding of antibodies to $E^{rns}_{aa\ 109–145}$, possibly by masking or altering the epitopes within the consensus region. Use of relatively smaller fragments, AR23 and AR2, both of which contain $E^{rns}_{aa\ 109–145}$, detected a similar number of antibody responses in pigs after infection compared to use of $E^{rns}_{aa\ 109–145}$. This provides further support that additional N-terminal amino acid sequences interfere with binding of antibodies to $E^{rns}_{aa\ 109–145}$.

The 20 serum samples from infected pigs used in the study cover various days postinfection (Table 1). Inspection of the ELISA data (OD_{414}) associated with each of the 20 serum samples (Fig. 3, 4, and 5) has allowed us to make the interesting observation that AR2, AR23, $E^{rns}_{aa\ 109–145}$, and $E^{rns}_{aa\ 109–160}$ tend to detect a nearly full spectrum of antibody responses, including those during early infection. In particular, antibody responses to AR23 or $E^{rns}_{aa\ 109–160}$ can be detected as early as 7 dpi. These small E^{rns} fragments offer an advantage over the entire protein, which can only be used to detect CSFV-specific antibodies in vaccinated or unvaccinated pigs as early as 14 dpi (21). None of the nine defined E^{rns} fragments was capable of detecting anti-E^{rns} antibodies in sera S#5 and

S#20 of infected pigs. These two sera were collected in early infection (8 days postchallenge [dpc] and 7 dpi), and most likely anti- E^{rns} antibodies had not yet been induced. Other E^{rns} fragments tend to detect only antibodies arising in the later stages of infection. Exploration of this striking difference will allow development of an E^{rns} fragment-based ELISA for the serological detection of CSFV infection at various stages of infection. Selection of a small E^{rns} fragment, such as $E^{rns}_{aa\ 109–145}$ and $E^{rns}_{aa\ 109–160}$ or a synthetic peptide corresponding to $E^{rns}_{aa\ 109–145}$, would offer a diagnostic antigen superior to the full-length protein. Production of recombinant E^{rns} fragments in large quantity from this study will facilitate the development and validation of a test that can be used for serological survey in a CSF-free country (e.g., Canada) in biocontainment level 2 laboratories.

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