Several Recombinant Capsid Proteins of Equine Rhinitis A Virus Show Potential as Diagnostic Antigens

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Equine rhinitis A virus (ERAV), formerly known as equine rhinovirus 1, is an important respiratory pathogen of horses worldwide. It has been shown to be responsible for outbreaks of acute respiratory disease in horses (11). Disease is characterized by fever, anorexia, nasal discharge, coughing, pharyngitis, and lymphadenitis (18). ERAV is classified with Foot-and-mouth disease virus (FMDV) in the genus Aphthovirus of the Picornaviridae family (10, 19, 20, 24, 28). The genome for all picornaviruses is single-stranded, positive-sense RNA containing a single open reading frame that encodes the capsid proteins. Studies on the three-dimensional structures of the capsid proteins of many picornaviruses, including FMDV, have revealed that picornaviruses share a great degree of structural homology and the major capsid proteins VP1, VP2, and VP3 share common folding patterns. Each is composed of a wedge-shaped, eight-stranded β-barrel which differs in the size and conformation of the connecting loops between the strands and the extensions of the N and C termini (14).

As the only two members in the Aphthovirus genus, ERAV and FMDV share many physicochemical and biological properties, as well as considerably similar genome structures and sequences (10, 16, 18, 28). A number of antigenic sites within capsid proteins VP1, VP2, and VP3 of FMDV, which contain neutralization epitopes, have been identified. Structural studies of these epitopes have shown that many are conformational (14). Recently, other FMDV-specific linear B-cell epitopes have also been identified, with some located in the nonstructural proteins (6).

In contrast to that of FMDV, the amino acid sequence of the ERAV structural region, VP1 in particular, is remarkably stable among different virus isolates (26). To date, studies on the antigenicity of ERAV have focused on the capsid protein VP1.

We have reported that ERAV VP1 contains B-cell epitopes that elicit neutralizing antibodies in rabbits and has receptor-binding activity (5, 27) and that regions at the N (VP1-NT) and C termini (VP1-CT) as well as the βE-βF and βG-βH loop regions of VP1 and the N terminus of VP3 contain nonneutralizing B-cell epitopes (23). More recently, the first neutralizing epitope of ERAV was identified and is thought to be formed by the quaternary structure of the viral capsid, where the C terminus of VP1 in each protomer extends to the βE-βF loop of VP1 on the adjacent protomer (9). Despite these findings, the kinetics of antibody responses following ERAV infection have not been clearly revealed, and the antigenic regions within the other capsid proteins, VP2 and VP3, are poorly understood. Together, these outcomes have meant that the diagnosis of ERAV infection continues to rely on virus isolation and serum neutralization assays using paired samples.

In this study, we expanded our study on the antigenic structure of the ERAV capsid proteins to VP2 and VP3, aiming to gain more information on the antigenic sites in ERAV and combine our existing knowledge of the antigenic structure of ERAV to identify potential antigens for a diagnostic assay. Recombinant full-length and truncated VP1, VP2, and VP3 were expressed in Escherichia coli and their antigenicities ex-
amined by using sera from experimentally infected and naturally exposed horses. Regions containing major B-cell epitopes within VP1 and VP2 were mapped, and the kinetics of the corresponding antibody responses were analyzed. The detection of ERAV antibody by use of the recombinant proteins was compared with assays using ERAV virus. The potential of these recombinant proteins for use as diagnostic antigens is discussed.

**MATERIALS AND METHODS**

**Cells and virus.** Vero cells were grown in minimal essential medium (GIBCO) containing salts, L-glutamine, and nonessential amino acids and supplemented with 50 μg/ml of ampicillin and 5% fetal calf serum. The ERAV 393/76 isolate used in this study and the protocol for virus purification were described previously (5, 24). The purity and yield of the virus were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining and Western blotting (WB) with rabbit anti-ERAV serum (see below) (5). Assays for virus-neutralizing (VN) antibodies were carried out in 96-well cell culture plates according to standard procedure in the laboratory (24, 27).

**Test sera.** Serial serum samples were obtained from five horses (G, C, B, S, and I) that were experimentally infected with ERAV 393/76 (5) for up to 105 days postinfection. In experiments in which pooled sera were used, sera from horse G and horse C from day 0 (prebleed) or from days 41 and 29 postinfection, respectively, were pooled. Serum samples referred to as “field sera” were also used in this study and comprised 12 sera that were submitted to the Centre for Equine Virology for testing for various purposes between 1995 and 1997. All serum samples were stored at −20°C.

**Construction of ERAV plasmids.** A schematic representation of the fusion proteins used in this study is shown in Fig. 1A. The pGEX-VP1.CT plasmid was constructed as described previously (23). To facilitate the purification of insoluble proteins, pQE vector (Novagen) was used for construction of plasmids pQE-VP1, pQE-VP2, pQE-VP2.2, pQE-VP2.4, and pQE-VP3 to produce fusion proteins with an N-terminal hexahistidine tag. Full-length VP1, VP2, and VP3 and VP2.2 and VP2.4 were amplified from the purified RNA of ERAV 393/76 by reverse transcription-PCR using primers containing BamHI (VP2, VP2.2, VP2.4, and VP3; upstream), SacI (VP1, upstream), or HindIII (downstream) restriction sites, respectively. The PCR fragment was digested with the respective restriction enzymes and ligated with pQE vector. The clones were screened by PCR and restriction enzyme digestion and confirmed by sequencing. The VP2-NT fragment was cloned into pGEX vector by use of a strategy similar to that for generating the plasmid pGEX-VP1.CT.

**Expression and purification of ERAV proteins.** Recombinant fusion proteins encoded by plasmids were expressed in *E. coli* and cells processed to separate fractions containing soluble and insoluble proteins according to a procedure described previously (13, 27). For proteins expressed by the pQE system, the insoluble proteins were resuspended in lysis buffer containing 8 M urea and incubated at 4°C for 30 min, followed by centrifugation for 15 min. The supernatant containing soluble proteins in urea was collected. The proteins were purified with Ni-nitrilotriacetic acid nickel-charged resin (QIAGEN) under a denaturing condition (8 M urea) according to a previously published procedure (12). The elution fractions collected were examined by SDS-PAGE and quantitated by using protein assay dye reagent (Bio-Rad) and stored in aliquots at

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**FIG. 1.** (A) Schematic representation of the ERAV capsid proteins in this study. Specific ERAV proteins were fused with either GST or hexahistidine (6xHis). Locations of the proteins derived from VP2 are indicated according to amino acid (aa) numbering of VP2. (B) Coomassie blue-stained SDS-PAGE of purified recombinant fusion proteins and purified ERAV virus.
RESULTS

Expression of the ERAV capsid proteins. A total of eight recombinant fusion proteins, full-length VP1, VP2, and VP3 and VP1-CT, VP2-NT, VP2.2, VP2.3, and VP2.4, were expressed in E. coli and purified via either GST or hexahistidine-tagged protein. Two control proteins, GST produced by pGEX-4T vector and QE2.1, an unrelated hexahistidine-tagged protein. Two control proteins, GST produced by pGEX-4T vector and QE2.1, an unrelated hexahistidine-tagged protein, were also produced in parallel. VP2.3 protein was insoluble and did not react with antiserum from the rabbit immunized with inactivated ERAV 393/76 in WB (results not shown) and was excluded from later experiments. The estimated molecular weights of the purified protein products were consistent with the predicted sizes of the proteins, including their respective tags (Fig. 1B). Some of these proteins presented as doublets, with VP2 also containing smaller forms and VP3 a much larger form of proteins. WB with sera immunized with inactivated ERAV 393/76 in WB (results not shown). The smaller forms most likely represent degraded or truncated products and larger forms aggregated product. As described previously, purified ERAV showed three major bands in SDS-PAGE, with molecular masses of approximately 26, 25, and 22 kDa, representing VP2, VP1, and VP3, respectively (Fig. 1B) (5).

Recombinant capsid protein antigens contain authentic B-cell epitopes. Serial serum samples from five horses experimentally infected with ERAV 393/76 were used to evaluate the antigenicities of the recombinant ERAV capsid proteins and the kinetics of the immune responses of horses against these antigens. It is noted that VN antibodies in each horse were detected at approximately 3 weeks postinfection (5).

The titers of pooled preinfection and postinfection sera from two horses (G and C) against each of the recombinant proteins in an ELISA. Titrations of pooled prebleed and postinfection sera from experimentally infected horses G and C against different recombinant ERAV capsid proteins and purified ERAV virus by ELISA. Plates were coated with 0.025 μg per well of individual antigen. Bound antibody was detected with horseradish peroxidase-conjugated goat anti-horse IgG and developed with 3,3′,5,5′-tetramethylbenzidine substrate. Therefore, with a 1/1,000 dilution of horseradish peroxidase-conjugated goat anti-horse immunoglobulin G (IgG) (KPL) in sample diluent for an additional hour. After each incubation step, the plates were washed four times with phosphate-buffered saline containing 0.05% Tween 20. The 3,3′,5,5′-tetramethylbenzidine substrate (Sigma) was added into the wells, and the plates were incubated for 5 min at room temperature. The reaction was stopped by the addition of 50 μl 1 M HCl, and the absorbance was read at 450 nm (Labsystems Multiskan MS). To control the background caused by some horse sera, an ELISA using control protein QE2.1, a protein unrelated to ERAV and expressed in the pQE system, or GST alone, was also performed in parallel to that for ERAV-specific proteins. The absorbance value obtained for each test serum against QE2.1 or GST was subtracted.

A blocking ELISA was performed to evaluate the proportions of the contributions of individual proteins to the reactivity of full-length VP1, VP2, and whole virus. Sera from experimentally infected horses G and C diluted 1/100, 1/200, and 1/400 were preincubated with individual antigens at a final concentration of 5 μg/ml at 37°C for 1 h before being added into protein- or virus-coated wells for an additional hour. The absorbance value obtained for each test serum against QE2.1 or GST was subtracted.
VP2, VP2-NT, gave higher postinfection/prebleed ratios. VP3 and VP2.2 gave relatively high background levels with the prebleed sera. By comparison, VP2.4 comprising the C-terminal region of VP2 showed similar reactivity to the preinfection sera and the postinfection sera (result not shown), suggesting that this region may not contain strong ERAV-specific epitopes. The antibody titration curve of purified virus was flatter than the ones of recombinant proteins; however, the background with the prebleed was low, and the end-point titer of ERAV antibody was the highest among all the antigens studied. These data suggest that the recombinant ERAV capsid proteins VP1, VP2, and VP3 present authentic viral epitopes and can be used to detect ERAV-specific antibodies in experimentally infected horse sera in an ELISA. The specific epitopes within VP2 may locate towards the N terminus rather than the C terminus.

Kinetics of the antibody response after ERAV infection. To examine the kinetics of the antibody response in experimentally infected horses, serial serum samples from five experimentally infected horses were tested for antibodies against each of the recombinant capsid proteins and purified ERAV in an ELISA. As presented in Fig. 3, VP1 and VP2.2 reacted with antibodies in sera from four of five horses, whereas VP1-CT, VP2, VP2-NT, and whole virus reacted with antibodies in sera from all five horses. VP3 reacted with antibodies in only three of the horses. Seroconversions of these antibodies were detected at approximately 3 weeks postinfection, consistent with the appearance of VN antibodies (5). It was noted that the levels of antibodies to VP1 and VP1-CT in horses G and S were in decline at 40 days postinfection.

For horses G and S, sera were collected up to 105 days in order to allow the kinetics of the antibody responses to be monitored over a longer time. The kinetics of VP1-, VP1-CT-, VP2-, and VP2-NT-reactive antibodies in these two horses showed different patterns. VP1 antibody peaked rapidly and then declined 1 month postinfection, whereas VP2-reactive antibody persisted over 3 months (Fig. 4A). Interestingly, almost identical patterns were also observed with individual viral capsid proteins from purified ERAV in WB with horse sera (Fig. 4B), further supporting the notion that recombinant VP1 and VP2 contain some ERAV-specific epitopes that are also present in the virus. The fact that we purified these proteins under a denaturing condition suggests that these epitopes are most likely linear and not conformational.

Major B-cell epitopes exist within the C terminus of VP1 and the N terminus of VP2. The similarity of the kinetics of the antibody responses between full-length VP1 and VP1-CT and the similarity of the kinetics of the antibody response between full-length VP2 and VP2-NT imply that the reactivity of VP1 or VP2 may largely represent that of the epitopes within its C terminus or N terminus, respectively (Fig. 4A). To further examine the contributions of specific epitopes within different regions of VP1 or VP2 to the reactivities of their recombinant full-length proteins, a blocking ELISA in which dilutions of test sera were preincubated with individual antigens before binding to the full-length proteins used to coat ELISA plates.

**FIG. 3.** Kinetics of the antibody response against different recombinant ERAV capsid proteins and purified ERAV virus up to 56 days postinfection for five experimentally infected horses (G, C, B, S, and I) by ELISA.
was performed. The results clearly showed that VP1-CT contributed approximate 70% of the reactivity of VP1, whereas the N-terminal region of VP1, VP1-NT, contributed only 15% despite also containing strong B-cell epitopes (Fig. 5) (23). For VP2, its N-terminal region, VP2-NT, accounted for over 60% of its reactivity, and the adjacent region covering β1 to βE, VP2.2, accounted for 35% of the reactivity of VP2 (Fig. 5). These data strongly suggest the presence of major B-cell epitopes within these regions, which are exposed on the recombinant VP1 and VP2 proteins.

The results from the blocking ELISA on the virus-coated plate were interesting in that they were distinct from the results shown for the individual capsid proteins. Figure 5 shows that virus itself does not significantly block the reactivity of the horse serum diluted at 1/100 to virus which was used to coat the ELISA plate, and blocking became more efficient when sera were diluted further. This finding implies that the virus may undergo some conformational changes when used to coat the ELISA plate and that different epitopes which are not present when the virus is in solution may be exposed. Recombinant proteins VP1 and VP1-CT showed minor blocking effects with sera diluted at 1/200 and 1/400 compared to VP2 and VP2-NT. However, it is difficult to draw any firm conclusion when the absorbance readings are low and differences are subtle.

Detection of ERAV antibodies in field equine sera. The antibody profiles in sera from 12 field horses were examined against virus antigens by using the VN assay, WB, and ELISA, and these results were compared with the results of detection of the antibody by using the recombinant antigens VP1-CT and VP2 in the ELISA. Both VP1-CT and VP2 were able to detect seroconversions in sera from all five experimentally infected horses (Fig. 3).

The VN assay is currently the standard method to detect ERAV-specific antibodies. As shown in Fig. 6A, six sera positive by VN clearly reacted with VP1, VP2, or VP3 from purified virus by WB; however, the intensity of the reactivity to these capsid proteins did not correlate with the VN titer. In
addition, WB identified two more ERAV antibody-positive horse sera (159 and 178) than were negative by VN, and these sera were clearly reactive against VP1 and/or VP2. By comparison, the results from the ELISA using intact purified virus corresponded to those from VN assay, and unlike the WB, the ELISA was unable to detect ERAV antibody in sera 159 and 178 (Fig. 6B). While both ELISA and VN generally detect antibodies to native epitopes exposed on the surface of the virus, the WB detects antibodies to denatured epitopes that may be not necessarily exposed on the surface. Interestingly, the absorbances of sera 159 and 178 increased to the levels of other VN-positive sera when inactivated virus was used as an antigen for the ELISA, indicating that these sera indeed contained antibodies against epitopes in denatured ERAV viral antigens (Fig. 6B).

Two recombinant proteins, VP1-CT and VP2, were used as antigens individually or combined in an ELISA and results compared with those from the VN assay and others using purified virus. In the ELISA using either VP1-CT or VP2, seven out of eight VN- or WB-positive sera gave higher absorbance values than the four VN- and WB-negative sera. Notably, the individual sera that failed to react were different for each antigen. The VP2 ELISA had a relatively high background, especially with one of the specific-pathogen-free (SPF) horses. Significantly, when the plates for ELISA were coated with both proteins, all eight field sera in the positive group had higher absorbances than those in the negative group, although the absorbance readings were generally low (Fig. 6B). These preliminary data suggest that VP1-CT and VP2 proteins are promising candidates for the further development of diagnostic ELISA for ERAV infection.

**DISCUSSION**

The close relationship between ERAV and FMDV and ERAV’s own significance as a pathogen in horses highlight the importance of better understanding the antigenic sites and the antibody responses to ERAV infection. To date, much of the study of the antigenic structure of ERAV has focused on the capsid protein VP1, and several surface loop regions containing linear B-cell epitopes have been identified (23). In this study, we expand our knowledge of the antigenic structure of ERAV and use this information to identify candidate antigens that have potential in a diagnostic ELISA.

Our results show that as recombinant proteins expressed in *E. coli*, VP1, VP1-CT, VP2, VP2-NT, VP2.2, and VP3 react with sera from experimentally infected horses. Among these proteins, VP1-CT, VP2, and VP2-NT reacted with antibodies in sera from all five infected horses. Antibodies against epitopes presented on recombinant VP2 persisted for longer periods postinfection than those on recombinant VP1. Our data also suggest the presence of major B-cell epitopes within the C terminus of VP1 and the N terminus of VP2, which contribute to large proportions of the reactivities of recombi-
nant VP1 and VP2, respectively. In addition, the reactivities, in ELISAs, of these VP1 and VP2 recombinant proteins correlated well with results from a range of native antigen-based serological assays using sera from 12 naturally exposed horses. Taken together, the data show that these proteins are promising candidates for further development of a much-needed diagnostic ELISA for ERAV.

Antigenic sites of picornavirus capsid proteins, as mapped mainly by use of monoclonal antibody-resistant virus mutants, locate to the surface-exposed loops of the virus capsid (14), although further studies suggest that most of the capsid that is accessible to antibodies could participate in the formation of epitopes (3, 7, 14, 22). In this study, recombinant VP1, VP2, VP2.2, and VP3 were purified under a denaturing condition with a hexahistidine tag due to their insoluble nature. Therefore, their reactivities revealed here are most likely against ERAV-specific linear epitopes within these regions. Consistent with this notion, VP1-CT, comprising the C-terminal 17 amino acids of VP1, has been shown to contribute to most of the reactivity of VP1 and has a higher detection efficiency than the full-length capsid protein. As it exists on virus particles, this region not only contains this major linear B-cell epitope but is also involved in the formation of a highly conformational neutralizing epitope (9).

Although it is well known that many antigenic sites containing neutralizing epitopes locate within VP1, neutralizing antigenic sites in VP2 and VP3 of most picornaviruses, e.g., poliovirus, rhinovirus, FMDV, or mengovirus (2, 4, 8, 15, 17, 21, 25), have also been identified. Compared to the case with FMDV, virus, rhinovirus, FMDV, or mengovirus (2, 4, 8, 15, 17, 21, 25), genic sites in VP2 and VP3 of most picornaviruses, e.g., poliovirus, rhinovirus, FMDV, or mengovirus (2, 4, 8, 15, 17, 21, 25), have also been identified. Compared to the case with FMDV, there is relatively little amino acid variation among ERAV isolates (26). The amino acid sequences of the capsid proteins of ERAV are highly conserved among 10 isolates, except for a small region between the \( \beta A2 \) and \( \alpha Z \) loops of VP2 and in the \( \beta E-\beta F \) loop of VP1 (26). Our study reveals the existence of major B-cell epitopes in highly conserved regions at the C-terminal region of VP1 and at the N-terminal region of VP2, which broadly react with antibodies in sera from infected horses. VP2.2, which overlaps with VP2-NT for 23 amino acids, displayed similar reactivities to antibody in most of the infected horses. It is unclear whether their reactivities are against the same epitopes. Different from antibody to VP1-CT, antibodies against VP2 and VP2-NT are relatively long lasting, which would make these antigens useful for the detection of specific antibody for long periods of time after infection. Finer mapping of these regions will allow us to identify minimum length of antigen containing the strong antigenic epitope.

To develop a convenient and sensitive diagnostic ELISA for the detection of ERAV-specific antibodies, it is important to identify antigens that can be produced feasibly and able to detect most, if not all, ERAV infections in horses. Given that the current standard method for the detection of ERAV-specific antibodies is the VN assay and that VP1 has been shown to be a target of neutralizing antibodies (9, 27), it might reasonably be expected that VP1 or a neutralizing epitope therein should provide a candidate antigen. The largely insoluble nature of full-length VP1 makes it a protein that is difficult to produce in large quantities, and, when expressed with a histidine tag and purified under denaturing conditions, recombinant VP1 had relatively low reactivity that showed no correlation with VN titers in the ELISA (unpublished data). Recent studies on a model of ERAV capsid (9) and our work on antigenic structure suggest that neutralizing epitopes within VP1 are highly conformational, and it is unlikely that an epitope formed by quaternary folding of the capsid could be produced as a recombinant antigen unless virus-like particles were made. Therefore, antigens other than full-length VP1 have to be sought to fulfill the requirements. Studies on other viruses, e.g., parechovirus, found that the most suitable antigen for diagnosis may not necessarily elicit neutralizing antibody (1). It is, therefore, worthwhile to explore broader areas within ERAV capsid proteins and nonstructural regions in order to identify suitable antigenic proteins for diagnostic use.

Sera from both experimentally infected horses and field horses were used to investigate the diagnostic potential of a range of recombinant antigens. Assignment of positive and negative sera by preinfection and postinfection times is obvious for experimentally infected horses but is more difficult with sera from field horses with unknown infection and exposure histories. While the current standard method for the detection of ERAV-specific antibody is by VN, we further investigated the reactivities of these field sera against viral antigen in WB and ELISA. Using these three assays, there were 4 of the 12 sera negative by VN, WB, and ELISA. In addition, two sera negative by VN were positive by WB and positive by the ELISA using denatured virus (Fig. 6B). These horses, therefore, have most likely been infected with ERAV. Among all the assays used in this study, WB is likely to be more sensitive than either VN or ELISA, and we regard sera positive by either VN or WB as ERAV antibody positive. Among the eight recombinant antigens studied, recombinant VP1-CT and VP2/VP2-NT appear to be promising candidates for further development of a diagnostic ELISA. The preliminary detection of antibody in field horses also indicates that VP2 and VP1-CT are individually able to detect antibodies in seven of eight horses most likely infected with ERAV (Fig. 6B) and, combined, appear able to detect antibody in all eight. At this time, the two most promising recombinant antigens do not show as significant a distinction between positive and negative field sera as is required for a diagnostic assay, despite the clear distinction with the experimentally infected horse sera noted. Engineering an improved antigen by stringing multiple copies of VP1-CT and VP2-NT may enable coverage of a broader spectrum of specific antibodies following ERAV infection and provide more specific differentiation of positive and negative sera.

In summary, this study identifies these proteins and mapped regions in the ERAV capsid as promising candidates for further development of a diagnostic ELISA. To our knowledge, this is the first time capsid proteins of ERAV other than VP1 have been explored and potential antigens for a diagnostic ELISA proposed. The information obtained on the antigenicities of VP1, VP2, and VP3 and the kinetics of antibody response following ERAV infection also provide more insight on antigenic structure of the virus and immunodominant B-cell epitopes. Further characterization of these important proteins and optimization of their antigenicities as well as ELISA conditions are in progress.
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