Arch Virol (2008) 153: 351–356
DOI 10.1007/s00705-007-1094-y
Printed in The Netherlands

Archives of Virology

Brief Report

Characterization of equine arteritis virus particles and demonstration of their hemolytic activity

M. Veit[1], **A. Kabatek**[1], **C. Tielesch**[1], **A. Hermann**[2]

[1] Department of Immunology and Molecular Biology, Veterinary Faculty, Free University Berlin, Berlin, Germany
[2] Institute of Biology, Humboldt-University, Berlin, Germany

Received 24 April 2007; Accepted 2 October 2007; Published online 4 February 2008

Summary

Equine arteritis virus (EAV), a member of the newly established family *Arteriviridae*, is a small, positive-stranded RNA virus. It carries two protein complexes in its envelope, gp5/M and the recently described gp2b/gp3/gp4 complex. We report here on several basic features of EAV replication in cell culture and on the protein composition of virus particles. We have also characterized gp2b, gp3, and gp4 expressed using a baculovirus system in insect cells. Finally, we provide evidence that EAV possess hemagglutinating and hemolytic activity. The hemolysis assay might be useful for determining which of the surface proteins carries the receptor-binding and membrane fusion activity of EAV.

*

Equine arteritis virus (EAV) is the prototype member of the family *Arteiriviridae*, which is grouped together with the coronaviruses in the order *Nidovirales* [14]. The positive-stranded RNA of EAV encodes seven structural proteins: the nucleocapsid protein N and six membrane proteins, the glycoproteins gp2b, gp3, gp4, gp5 and the unglycosylated proteins E and M. The membrane proteins are present as monomers (E) or form disulfide-linked heterodimeric (gp5/M) or heterotrimeric complexes (gp2b/gp3/gp4) [3, 4, 6, 15, 18–20]. All of the structural proteins are essential for virus infectivity, but their function during the replicative cycle of EAV is not known. Gp5 and M, but not E, gp2b, gp3, or gp4, are required for budding of virus particles at internal membranes, implicating the latter in virus entry [21]. Likewise, exchanging the ectodomains of gp5 and M of EAV with those of other arteriviruses does not change the cell tropism of the recombinant virus [5, 17]. Thus, the recently described gp2b/gp3/gp4 complex is currently a prime candidate for the receptor-binding and probably also the membrane-fusion activity of EAV.

Here, we have investigated several basic features of EAV, such as virus growth in cell culture and preparation of virus particles, and have analysed their structural proteins. Furthermore, we report for the first time that EAV particles exhibit hemolytic activity. This classical feature of many enveloped viruses [1] has been very useful in the

Correspondence: Michael Veit, Department of Immunology and Molecular Biology, Veterinary Faculty, Free University Berlin, Philippstraße 13, D-10115 Berlin, Germany
e-mail: mveit@zedat.fu-berlin.de

past for identifying proteins involved in cell entry of viruses.

We first compared several procedures for growth of EAV in BHK cells. Confluent monolayers were infected at a multiplicity of infection of 10, and cells were incubated at 37 °C or 39.5 °C for 14 or 22 h. Aliquots of the supernatant were then removed and the virus titer was determined using a plaque assay (Fig. 1A). Virus release was more efficient at 37 °C compared to 39.5 °C and more infectious virus was present when cell culture supernatants were harvested at 22 h postinfection. DEAE-dextran, which is thought to enhance virus binding to cells in the initial adsorption phase, slightly decreased vi-

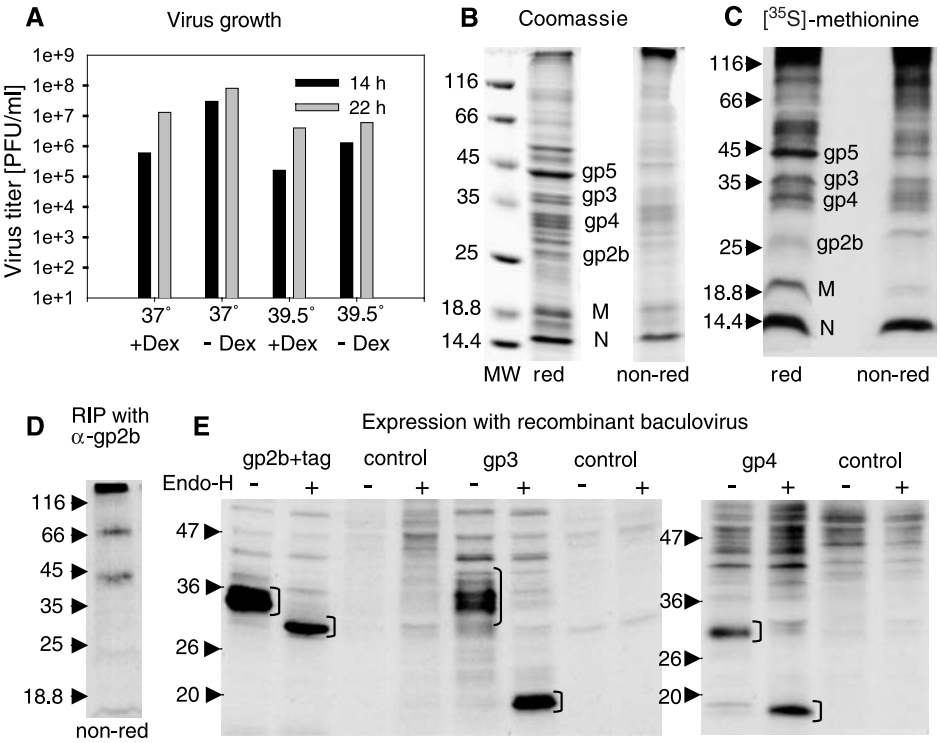

Fig. 1. Protein composition of virus particles and expression of gp2b, gp3, and gp4 using baculovirus. (**A**) Confluent monolayers of BHK cells were infected with EAV (Bucyrus) at a multiplicity of infection of 10 and were incubated for 14 or 22 h, either at 37 °C or 39.5 °C. The cell culture supernatants were removed and centrifuged (3000 RPM, 15 min), and the virus titer was determined using a plaque assay according to reference 8 except that plates were stained with neutral red 48 h after infection. Samples labeled with "Dex" contained DEAE-dextran (50 µg/ml) during the two-hour adsorption period. (**B**) BHK cells were infected with EAV and incubated for 22 h at 37 °C. Cell debris was removed from cell culture supernatants by low-speed centrifugation (3000 × *g*, 15 min), and virus particles were pelleted (28,000 RPM, 2 h). The virus pellets were resuspended in phosphate-buffered saline, and aliquots were subjected to SDS-PAGE (12% gel) using reducing or non-reducing conditions and staining with Coomassie blue. *MW* Molecular weight markers. (**C**) EAV-infected cells were labeled with 10 µCi/ml [^{35}S]-methionine/cysteine starting at 5 h postinfection. Virus particles prepared as described above were subjected to SDS-PAGE and fluorography. (**D**) [^{35}S]-labeled EAV particles were lysed in RIPA buffer (1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate, 0.15 M NaCl, 20 mM Tris, 10 mM EDTA, and 10 mM iodoacetamide), and were subjected to immunoprecititation with 5 µl of peptide antiserum α-gp2b [2], followed by SDS-PAGE under non-reducing conditions and fluorography. (**E**) Gp2b equipped with a His- and a FLAG-tag at the C-terminus and authentic gp3 and gp4 were expressed in Sf9-cells by recombinant baculovirus, which was produced as described by the manufacturer ("BaculoDirect", Invitrogen). Cells labeled with [^{35}S]-methionine/cysteine (10 µCi/ml) for 4 h were lysed with RIPA buffer, and cellular extracts were subjected to immunoprecipitation with 5 µl of peptide antiserum α-gp2b or peptide antiserum α-gp3 or antiserum α-gp4 [18]. Samples were then split into two aliquots, which were digested with 1 µl Endo-H (+, New England Biolabs) or mock-digested (−) for 1 h prior to SDS-PAGE and fluorography. Control: cells infected with non-recombinant baculovirus

rus production. The highest virus titer, i.e. 1×10^8 plaque-forming units/ml, was achieved with virus growth for 22 h at 37 °C.

We analyzed the virus particles pelleted from supernatants of infected BHK cells by SDS-PAGE, both by Coomassie staining (Fig. 1B) and by metabolic labeling of their proteins (Fig. 1C). Especially the Coomassie staining of the virus preparations showed that they were contaminated by cellular proteins. Attempts to purify the virus preparation further by precipitation of cell culture supernatants with polyethylenglycol (10% PEG 6000) or by centrifugation through a 20% sucrose cushion yielded identical results. Furthermore, loading the virus preparation onto a continuous sucrose gradient did not generate a detectable virus band (data not shown).

Nevertheless, the viral gene products are the major proteins in our preparation, which resemble those published previously [9]. Two major protein bands were present at the bottom of the gel, with molecular weights of 15 and 19 kDa, corresponding to the nucleocapsid protein N (predicted MW of 12,300) and the matrix protein M (predicted MW 17,700). Another major band was visible at 42 kDa, which likely represents the fully glycosylated form of gp5 [2]. M and gp5 are known to from disulfide-linked complexes in virus particles, whereas N is monomeric. Indeed, separation of the virus preparation under non-reducing conditions shifted the M and gp5 bands (but not N) to positions corresponding to higher molecular weights not resolved in the gel. Between gp5 and M, a variety of bands are apparent, representing gp2b, gp3, and gp4. These bands also shifted to higher-molecular-weight positions when SDS-PAGE was employed under non-reducing conditions. To unequivocally demonstrate the presence of disulfide-linked complexes, we subjected aliquots of [^{35}S]-methionine-labeled EAV particles to immunoprecipitation with antibodies against gp2b. SDS-PAGE under non-reducing conditions revealed bands with molecular weights of 45 and 66 kDa (Fig. 1D), which represent gp2b/gp4 dimers and gp2b/gp3/gp4 trimers, as described previously [20].

In order to set up a system allowing high-yield production of viral proteins, we characterized the gp2b, gp3, and gp4 proteins further by expressing their genes using a baculovirus system in insect cells. Gp3 and gp4 were expressed as native, full-length proteins, whereas gp2b was equipped with a FLAG-His-tag at its cytoplasmic tail to possibly allow its purification. Sf9 cells infected with recombinant baculovirus were labeled with [^{35}S]-methionine, and cellular extracts were subjected to precipitation with antibodies against the viral glycoproteins. An aliquot of the immunoprecipitate was digested with endoglycosidase H (+) to analyze proteins for glycosylation (Fig. 1E). SDS-PAGE and fluorography showed that gp3 was visible as multiple bands with molecular weights between 28 and 38 kDa. They shifted after Endo-H digestion to a single band with a molecular weight of 18 kDa, indicating that the protein with its five putative glycosylation sites is heavily and heterogeneously glycosylated, as has been observed before after expression of the gp3 gene in BHK-cells [18]. Gp4 was expressed as a single band with a molecular weight of 30 kDa, which is present as an 18-kDa band after Endo-H cleavage, indicating that all of its four potential glycosylation sites are filled. Due to the attached His-FLAG tag, gp2b is expressed with a molecular weight of 34 and 6 kDa heavier than the native protein. Upon Endo-H digestion its molecular weight was slightly reduced, consistent with the presence of only one glycosylation site. These results are in agreement with the sizes of gp2b, gp3 and gp4 in virus particles (compare with Fig. 1C) and with published studies on the expression of gp2b, gp3, and gp4 in mammalian cells [4, 18], indicating that the baculovirus system is able to generate authentic EAV glycoproteins. However, their expressions levels were disappointingly low, and neither gp2b alone nor – upon triple infection of SF9 cells – a gp2b/gp3/gp4 complex could be purified by nickel-affinity chromatography (data not shown).

It has been described that extracts prepared from EAV-infected cells, especially when they were treated with Tween/ether, can hemagglutinate chicken erythrocytes with titers up to 64 [10, 13]. Our virus preparations (50 µl [1–10 µg/µl protein] incubated with 50 µl of 1% erythrocytes at room temperature) usually exhibited HA titers between 8 and 16. Does EAV also possess hemolytic activity, which

would indicate that it carries surface proteins with membrane fusion activity? Incubation of erythrocytes with EAV indeed caused release of hemoglobin, and we observed a linear dependency between the virus concentration and hemoglobin release (Fig. 2A). It was not possible to increase the virus concentration further to analyze whether hemoglobin release reaches saturation. The hemolytic activity of EAV is rather low compared to that of other viruses. Electron microscopy of virus preparations showed many virus particles with disrupted membranes, as described before [9]. Likewise, the virus preparations exhibited lower titers (1×10^9 pfu/ml) than one would expect for a more than 100-fold concentrated virus pellet (compared to the cell culture supernatant). Thus, EAV particles are fragile, and their infectivity and membrane fusion activity is partly destroyed during centrifugation.

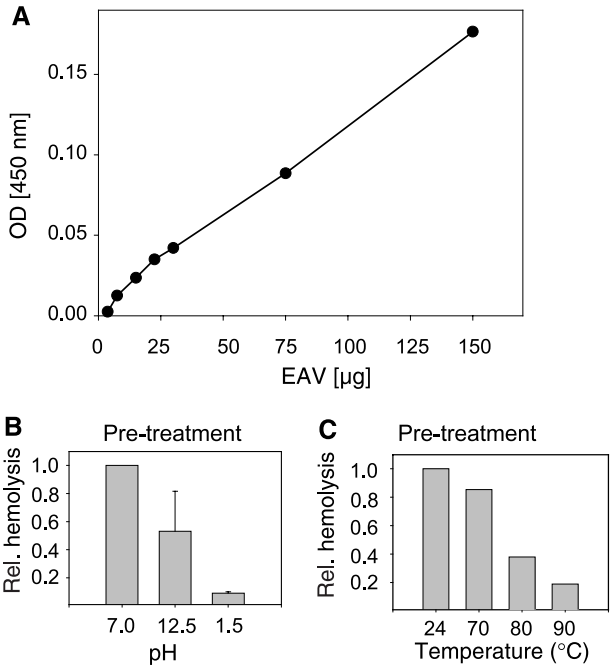

Fig. 2. EAV exhibits hemolytic activity. (**A**) Increasing amounts of EAV prepared as described in Fig. 1b were incubated with 0.5% chicken erythrocytes in a final volume of 50 µl citrate-saline buffer (20 mM citrate (pH 7), 120 mM NaCl) at 37 °C for 60 min. Erythrocytes were then pelleted at $600 \times g$ for 10 min, and the optical density (OD) of the supernatant was measured at 450 nm. The OD 450 of samples incubated without virus was subtracted. The mean of triplicate samples analyzed in parallel is shown. Twenty microgram of EAV corresponds to a protein concentration of 400 µg/ml in the assay. (**B**) Virus preparations (20 µg) were incubated with 1 µl 1 M NaOH (pH 12.5) or 1 µl 1 M HCl (pH 1.5) for 15 min at room temperature. The pH was then adjusted to neutral values by adding 1 µl HCl and 1 µl 1 M NaOH, respectively, prior to performing the assay. The mean of three independent experiments (duplicates) including the standard deviation is shown. The highest OD 450 was set to 1 for each experiment. (**C**) Virus preparations (20 µg) were incubated for 10 min at 24 °C, 70 °C, 80 °C or 90 °C, briefly centrifuged ($1000 \times g$, 1 min), and cooled on ice prior to performing the assay. The highest OD 450 was set to 1

We asked whether conditions known to denature proteins might inhibit the hemolytic activity of EAV. Shifting the pH to acidic (1.5) or to basic values (12.5) prior to performing the assay eliminated or clearly reduced hemolysis (Fig. 2B). Likewise, pre-treatment of virus particles at 90 °C abolished their hemolytic activity, although it is remarkably stable at 70 °C (Fig. 2C). Furthermore, trypsin treatment (1 µg/µl, 10 min) of the virus preparation prior to incubation with erythrocytes reduced release of hemoglobin to 50% (not shown). Thus, hemolytic activity of the virus preparations requires proteins. Incubation of EAV with increasing erythrocyte concentration did not increase the amount of released hemoglobin (Fig. 3A), indicating that the erythrocyte concentration is not limiting. Because the absolute values for OD 450 are rather low, we calculated the percentage of erythrocytes lysed by EAV relative to complete hemolysis accomplished with a detergent. At a 1% erythrocyte concentration this percentage is low (10%), but it increases up to 70% at an erythrocyte concentration of 0.1% (Fig. 3B). Finally, we asked whether performing the assay at mildly acidic pH might increase the hemolytic activity of EAV. This has been described for many viruses, such as influenza virus and togaviruses, which enter cells *via* the endocytic pathway, and this phenomenon reflects activation of the viral fusion protein at the acidic pH of the endosome [7, 12, 16]. However, we did not observe an increase in the hemolytic activity of EAV at acidic pH (Fig. 3C). Thus, in this respect, EAV resembles paramyxoviruses, which fuse with the plasma membrane [11]. Nevertheless, we do not want to exclude that EAV enters cells *via* the endo-

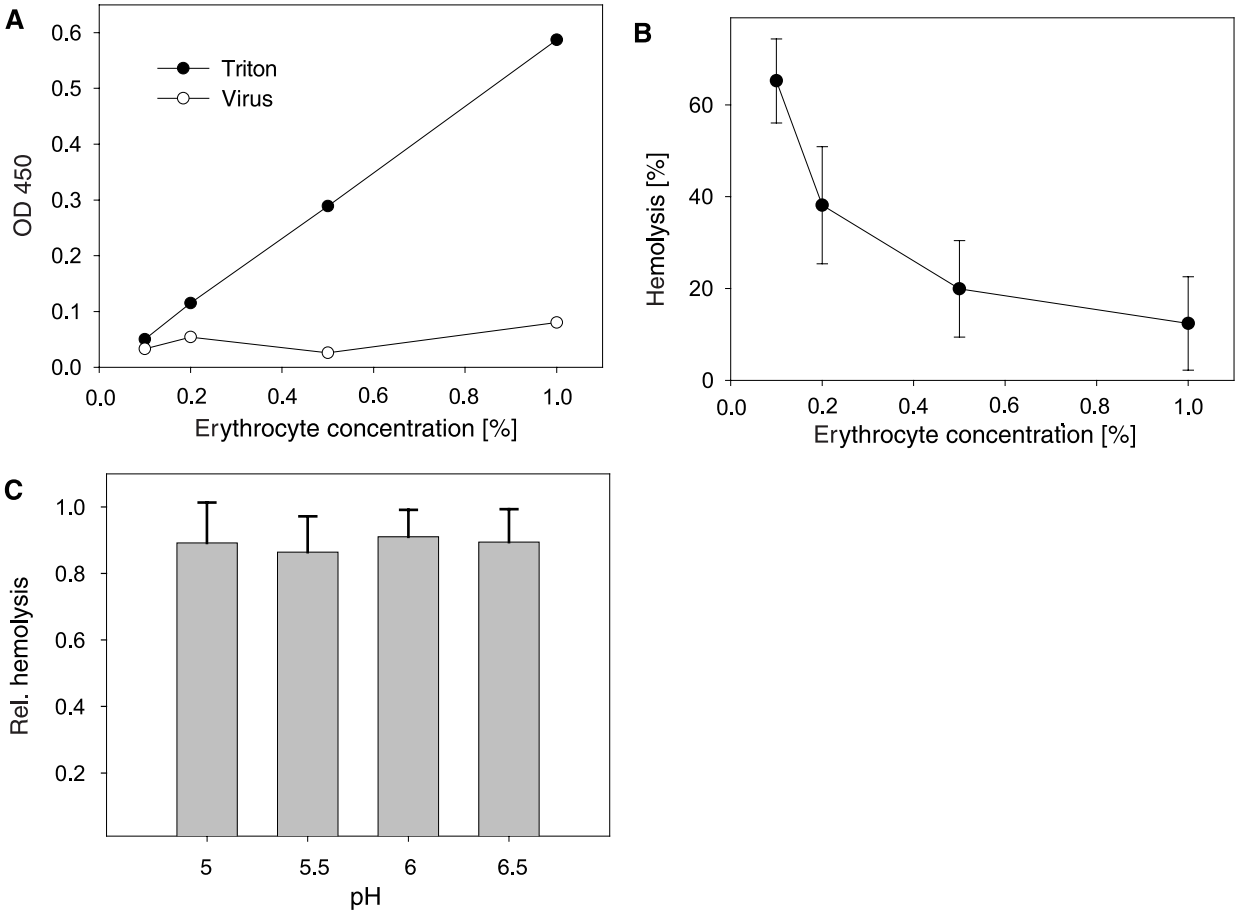

Fig. 3. Characterization of the hemolytic activity. (**A**) Twenty microgram of EAV was incubated in citrate-saline buffer with 0.1, 0.2, 0.5 or 1% erythrocytes. To achieve complete hemolysis, erythrocytes were incubated with 1% Triton-X-100 instead of virus. The mean of triplicate samples analyzed in parallel is shown. (**B**) Twenty microgram of EAV were incubated in citrate-saline buffer with 0.1, 0.2, 0.5 or 1% erythrocytes. Percent hemolysis (OD 450 for Triton X-100 lysis = 100%) is plotted against the erythrocyte concentration. The mean of three independent experiments done in duplicate, including their standard deviation, is shown. (**C**) Twenty microgram of EAV was incubated with 0.5% erythrocytes in citrate-saline buffer adjusted to the indicated pH. The mean of three independent experiments done in duplicate, including the standard deviation, is shown. The highest OD 450 of each experiment was normalized to 1

cytic pathway. We have observed that pre-treatment of cells with lysosomotropic agents inhibits virus replication (Nitzschke et al., in preparation). The membrane proteins of EAV involved in virus entry as well as their receptor on the target cell have not been identified. It might be that EAV, in principle, can fuse at neutral pH, but its cellular receptor is rapidly endocytosed, and subsequent acidification in the endosome is required to release virus particles from the receptor. Furthermore, we have to note that hemolytic activity may not correlate with membrane fusion activity. Once surface proteins of EAV have been purified, the hemagglutination and hemolysis assay described here might be useful for determining which complex (e.g. gp5/M or gp2b/gp3/gp4) contains the receptor-binding and membrane fusion activity of EAV.

Acknowledgement

We thank Peter Rottier (Virology Division, Faculty of Veterinary Medicine, Utrecht) for providing EAV seed virus, BHK cells, antisera, and cloned genes. This project is funded by the DFG through a joint grant to MV and AH (Ve 141/6 and He 1928/10).

References

1. Burnet FM (1949) Haemolysis by Newcastle disease virus. Nature 164: 1008
2. de Vries AA, Chirnside ED, Horzinek MC, Rottier PJ (1992) Structural proteins of equine arteritis virus. J Virol 66: 6294–6303
3. de Vries AA, Post SM, Raamsman MJ, Horzinek MC, Rottier PJ (1995) The two major envelope proteins of equine arteritis virus associate into disulfide-linked heterodimers. J Virol 69: 4668–4674
4. de Vries AA, Raamsman MJ, van Dijk HA, Horzinek MC, Rottier PJ (1995) The small envelope glycoprotein (GS) of equine arteritis virus folds into three distinct monomers and a disulfide-linked dimer. J Virol 69: 3441–3448
5. Dobbe JC, van der Meer Y, Spaan WJ, Snijder EJ (2001) Construction of chimeric arteriviruses reveals that the ectodomain of the major glycoprotein is not the main determinant of equine arteritis virus tropism in cell culture. Virology 288: 283–294
6. Hedges JF, Balasuriya UB, MacLachlan NJ (1999) The open reading frame 3 of equine arteritis virus encodes an immunogenic glycosylated, integral membrane protein. Virology 264: 92–98
7. Huang RT, Rott R, Klenk HD (1981) Influenza viruses cause hemolysis and fusion of cells. Virology 110: 243–247
8. Hyllseth B (1969) A plaque assay of equine arteritis virus in BHK-21 cells. Arch Gesamte Virusforsch 28: 26–33
9. Hyllseth B (1973) Structural proteins of equine arteritis virus. Arch Gesamte Virusforsch 40: 177–188
10. Kubota T, Inaba Y, Uwatoko K, Akashi H, Fukunaga Y (1997) Hemagglutination with equine arteritis virus. J Vet Med Sci 59: 943–945
11. Lamb RA, Paterson RG, Jardetzky TS (2006) Paramyxovirus membrane fusion: lessons from the F and HN atomic structures. Virology 344: 30–37
12. Maeda T, Ohnishi S (1980) Activation of influenza virus by acidic media causes hemolysis and fusion of erythrocytes. FEBS Lett 122: 283–287
13. Sano Y, Inaba Y, Uwatoko K, Kubota T, Asagoe T, Kanaya J, Pan IJ, Akashi H, Fukunaga Y (1998) Effect of heparin on hemagglutination by equine arteritis virus. J Vet Med Sci 60: 447–450
14. Snijder EJ, Meulenberg JJ (1998) The molecular biology of arteriviruses. J Gen Virol 79 (Pt 5): 961–979
15. Snijder EJ, van Tol H, Pedersen KW, Raamsman MJ, de Vries AA (1999) Identification of a novel structural protein of arteriviruses. J Virol 73: 6335–6345
16. Vaananen P, Kaariainen L (1979) Haemolysis by two alphaviruses: Semliki Forest and Sindbis virus. J Gen Virol 43: 593–601
17. Verheije MH, Welting TJ, Jansen HT, Rottier PJ, Meulenberg JJ (2002) Chimeric arteriviruses generated by swapping of the M protein ectodomain rule out a role of this domain in viral targeting. Virology 303: 364–373
18. Wieringa R, de Vries AA, Raamsman MJ, Rottier PJ (2002) Characterization of two new structural glycoproteins, GP(3) and GP(4), of equine arteritis virus. J Virol 76: 10829–10840
19. Wieringa R, De Vries AA, Post SM, Rottier PJ (2003) Intra- and intermolecular disulfide bonds of the GP2b glycoprotein of equine arteritis virus: relevance for virus assembly and infectivity. J Virol 77: 12996–13004
20. Wieringa R, de Vries AA, Rottier PJ (2003) Formation of disulfide-linked complexes between the three minor envelope glycoproteins (GP2b, GP3, and GP4) of equine arteritis virus. J Virol 77: 6216–6226
21. Wieringa R, de Vries AA, van der Meulen J, Godeke GJ, Onderwater JJ, van Tol H, Koerten HK, Mommaas AM, Snijder EJ, Rottier PJ (2004) Structural protein requirements in equine arteritis virus assembly. J Virol 78: 13019–13027