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Table 1 is included here; Tables 2–5 are in a separate file

Capsid protein sequence diversity of avian nephritis virus

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Abstract:

The capsid gene sequences of 25 avian nephritis viruses (ANV), collected in the UK, Germany and Belgium from the 1980s to 2008, were determined and compared with those of serotype 1 (ANV-1) and serotype 2 (ANV-2) ANV isolates. Amino acid identities as low as 51% were determined. Pairwise comparisons supported by phylogenetic analysis identified 6 ANVs, including ANV-1 and ANV-2, which shared <80% amino acid identities with one another, and which were selected to be representative of 6 groups. The ANVs were not distributed according to geographical location or year of sampling, and the detection of ANVs from 5 different groups in 11 samples sourced from 6 flocks belonging to the same UK organisation within a 4-month period indicated that sequence-diverse ANVs were co-circulating. Amino acid alignments demonstrated the existence of variable regions throughout the capsid protein, 9 of which were selected for detailed comparisons. With most ANVs, the variable region sequences were similar to those of one of the 6 representative ANVs, but some ANV capsids displayed novel variable region profiles, in which variable regions that were characteristic of more than one representative ANV were present. Phylogenetic analysis based on C-terminal sequences of approximately 260 amino acids and SimPlot analysis provided evidence that RNA recombination events located in the 1250-1350 nucleotide region resulted in new combinations of the N- and C-terminal capsid regions. The high level of capsid sequence diversity observed in this study has important implications for both the control and diagnosis of ANV infections.

Introduction

Avian nephritis virus (ANV), which is now classified as an avian astrovirus on the basis of its genome sequence (Imada *et al.*, 2000), was first isolated from the rectal contents of apparently normal 1-week-old broiler chickens (Yamaguchi *et al.*, 1979).

Diarrhoea, interstitial nephritis, lower body weights and mortality resulted when 1-day-old SPF chickens were experimentally infected with this isolate (Imada *et al.*, 1979; Maeda *et al.*, 1979). Serological studies performed in the 1980s, which were based on the detection of antibodies to the G-4260 serotype 1 ANV strain (ANV-1), showed that ANV infections were widespread in commercial chickens in Japan and in several European countries (Imada *et al.*, 1980; Connor *et al.*, 1987) and antibody to ANV was also detected in turkeys and in SPF flocks.

Although serological studies suggested that many ANV infections were subclinical, the detection of ANVs and enterovirus-like viruses (ELVs) that are antigenically related to ANV, hereafter designated ANVs, in broiler flocks affected by enteritis and growth problems, including infectious stunting or runting stunting (McNulty *et al.*, 1984; Decaesstecker *et al.*, 1986; 1989; Shirai *et al.*, 1992), and the demonstrations that experimental infections with these viruses resulted in significant degrees of growth retardation, have led to speculation that ANV may be important in causing growth problems in the field. However, the clinico-pathological and economic importance of ANV infections remains unknown largely because convenient diagnostic tests are lacking. ANVs grow relatively poorly in cell culture and are often difficult to isolate due to the frequent occurrence in enteric samples of more cultivable viruses such as reoviruses or adenoviruses. Reverse transcription polymerase chain reaction (RT-PCR) tests have recently been developed (Mandoki *et al.*, 2006b; Day *et al.*, 2007; Todd *et al.*, 2010; Smyth *et al.*, 2010 in press) and application of these tests confirmed the high prevalence of ANV infections in broiler chickens.

ANVs differ antigenically and at least 2 different serotypes have been detected. Based on indirect immunofluorescence (IIF) and virus neutralization (VN) tests, the G-4260 and IR-N isolates were assigned to the same serotype (serotype 1), while the M6 and M8 isolates were assigned to serotype 2 (Shirai *et al.*, 1991). Relatively low levels of IIF cross-reactivity were observed when representative isolates from each of these serotypes were compared. Further work by Shirai *et al.*

(1992), which showed that the WG3, WG4 and WG5 isolates shared low levels of IIF cross-reactivity and no cross-neutralization reactivity with either the G-4260 or M8 isolates, supported the view the WG3-5 isolates belonged to a third ANV serotype. ANV isolates may also vary with regard to pathogenicity and tropism. For example, the G-4260 isolate was found to differ from the M8 and WG5 isolates in terms of causing growth retardation and kidney lesions (Shirai *et al.* 1991; 1992). In comparison to the G-4260 ANV, the Belgian PV2 isolate, originally recognised as an ELV, was shown to cause substantial growth retardation and severe and long-lasting intestinal lesions following experimental infection (Decaesstecker *et al.*, 1989). The detection of lesions in the pancreas and proventriculus and their absence in the kidney tissue from chickens experimentally infected with PV2 suggested that some ANV isolates such as PV2 may have different tissue tropisms.

Comparatively little is known about the sequence diversity of ANV. The genome of only one isolate, the G-4260 serotype 1 isolate (ANV-1) has been completely sequenced (Imada *et al.*, 2000). Like its mammalian and avian astrovirus counterparts, the positive sense RNA genome of ANV contains 3 open reading frames (ORFs). ORF 1a, located close to the 5' terminus of the genome, encodes the viral (serine) protease and is followed by ORF 1b, which encodes the RNA polymerase. ORF 2, encoding the precursor capsid protein, is located after ORF 1b and prior to the 3' untranslated region (UTR) of the genome. Research with human astrovirus (HAstV) serotypes has shown that ORF 2 is translated to produce precursor capsid proteins of 87-90 kDa, which are proteolytically cleaved to produce multiple smaller proteins, but attempts to identify the number and size of the mature structural proteins have yielded discrepancies (Krishna, 2005). In the case of HAstV serotype 1, the 87kDa precursor capsid protein is cleaved to yield proteins of 34, 29 and 26 kDa, with the 34 kDa protein being derived from the N-terminal portion of precursor capsid and the 29 and 26 kDa proteins being derived from the C-terminal portion (Bass & Qiu, 2000).

In addition to the genomic sequence of ANV-1, a partial sequence of 2536 nucleotides (nt), which encompasses the capsid protein gene and the 3' UTR of the genome, is also available for a serotype 2 ANV isolate (ANV-2), and comparisons showed that the capsid protein genes of ANV-1 and ANV-2 share 69% nucleotide and 71% amino acid identities respectively. Pantin-Jackwood *et al.* (2006a) reported that 13 partial ORF 1b ANV sequences, which were detected in turkey and chicken

samples, shared 86.2-99.6% nucleotide and 92.4-100% amino acid identities, while 11 Hungarian ANVs detected in kidney samples obtained from broiler flocks affected by diarrhoea, gout and runting-stunting shared ORF 1a nucleotide and amino acid identities of 76-86% and 88-93% respectively with the reference ANV-1 isolate (Mandoliki *et al.* 2006a).

The aim of this study was to investigate the sequence diversity that exists within the ANV capsid protein gene. On the basis that it interacts with the host's immune system and host cell receptors, the astrovirus capsid protein is likely to be the key determinant of virus antigenicity and cell tropism, and, as such, it will influence pathogenesis and pathogenicity. In this paper we report the capsid protein gene sequences and sequence comparisons of 25 ANVs found in samples from broiler flocks with growth problems in the UK, Belgium and Germany from the 1980s to 2008, with those of ANV-1 and ANV-2 strains accessed from sequence databases.

Materials and Methods

Viruses and virus growth. The G-4260 serotype 1 isolate of ANV (ANV-1), obtained from Dr S. Yamaguchi, National Institute of Animal Health, Gifu, Japan, was propagated in chick kidney cells, with the virus pool used in this investigation having an infectious titre of 10^5 (median tissue culture infective doses) TCID₅₀/ ml. An ELV, designated Northern Ireland ELV 1, produced in this laboratory and present as an extract of intestinal contents (EF91-276), was derived from broiler chickens with runting-stunting syndrome (McNulty *et al.*, 1984). A second ELV, designated Belgian ELV 1, was supplied by Dr G. Meulemans, Brussels, Belgium, and was prepared as described previously (McNulty *et al.*, 1991). Cell culture lysates, clarified 10% whole embryo or embryo liver homogenates, or clarified 10% faeces/intestinal content suspensions were used for RNA extraction.

Sample origin and processing. Intestinal contents were sampled from chickens collected from problem flocks in the UK and Germany and stored at -80°C. Samples from the UK were kindly provided by A. Fernandez-Gutierrez (Aviagen Ltd., Midlothian, UK), M. Alcorn (St David's Poultry Team Ltd., Exmouth, UK), B.H. Thorp (St David's Poultry Team at the Dick Vet, Easter Bush Veterinary Centre,

Midlothian, UK), C. Prins, G. Hayes and A. Walker (Slate Hall Veterinary Practice Ltd., Cambridge, UK) and D. Pearson (VION UK Veterinary and Food Diagnostic Service, Grampian Country Chickens (Rearing) Ltd, West Lothian, UK). Eighty pooled samples were collected in longitudinal surveys of 4 broiler flocks as described previously (Todd *et al.*, 2010). Briefly, gut contents from approximately 12 birds were sampled from each flock at days 0, 4 or 5, 7, 10, 14, 21, 28 and 35 days. The samples were grouped into 4 pools and processed by homogenisation as described above. Intestinal contents were diluted 1:10 in chilled PBS and then shaken by hand briefly with glass beads to disrupt the solid material. Suspensions were centrifuged at 3000g at 4°C for 30 min, and the supernatants transferred to fresh tubes for storage at -80°C until required. Table 1 lists the samples investigated. Dr P. Otto (Friedrich Loeffler Institute, Federal Institute for Animal Health, Jena, Germany) provided samples 05V0105 and 06V0062.

RNA extraction. Viral RNA was extracted from 140µl of each supernatant using the QIAamp Viral RNA or Rneasy Mini Kits (Qiagen, Crawley, UK) according to the manufacturer's instructions. Each RNA was eluted in 30µl of RNase-free water.

RT-PCR & cloning, sequencing and sequencing analysis. Fragments of approximately 2.5 kbp were amplified from RNAs that had been extracted from the isolates and samples listed in Table 1 using the ANV 2.5kb forward primer, 5'- ACC TTG AAT CCC TGT GGG GCA - 3', and ANV 2.5kb reverse primer 5'-AAA AGT TAG CCA ATT CAA AAT TAA TTC - 3' in a single-tube RT-PCR format. Amplification conditions involved a reverse transcription step of 45°C for 30 min, an initial denaturing step of 94°C for 2 min, followed by 35-40 cycles each comprising denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec and extension at 68°C for 2.5 min. The amplified 2.5 kbp fragments, which corresponded in location to nucleotide 4379 to 6927 of the ANV-1 genomic sequence, were cloned using TOPO TA Cloning[®] Kit (Invitrogen). At least 2 representative recombinant plasmids, each containing one of the 2.5 kbp ANV-specific inserts, were sequenced using M13 forward and reverse primers followed by primer-walking strategies. If the M13 primer-derived sequences of the 2 cloned inserts differed by more than 2%, the sequences of both clones were fully determined, otherwise 1 cloned insert was sequenced for each sample. The products were sequenced commercially using the

BigDye[®] Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing data was analysed using the Vector NTI suite (Invitrogen). Phylogenetic analysis was conducted on the isolated ORF 2 (capsid) sequences using *MEGA* version 4 (Tamura *et al.*, 2007). Simplot analysis was undertaken with the capsid gene nucleotide sequences of selected ANVs using the method of Lole *et al.* (1999). This involved comparing the nucleotide sequences using a window of 200 nucleotides, which was moved along in 20-nucleotide steps, with the percent identities being calculated for each window and plotted in line charts.

Results

Cloning and sequence determination. Fragments of approximately 2.5kb were amplified using the ANV 2.5kb forward and reverse primer set from the majority of gut content RNA extracts, which had tested positive using the conventional or real-time ANV RT-PCR tests. This primer set was designed following consideration of the published ANV-1 and ANV-2 nucleotide sequences and the amplified fragment encompassed part (approximately 190 nt) of the RNA polymerase gene, the complete capsid gene, and the 3' untranslated region (3'UTR) of the ANV genomes. For 3 samples (EF91-276, VF08-3 and VF08-29), the sequences determined with either the forward or reverse M13 primer showed substantial variation and in these cases, 2 different clones were completely sequenced. In the case of VF08-3 and VF08-29 the clones were labelled "a" and "b", whereas, in the case of EF91-276 the clones were labelled "C13" and "C15". Data relating to the origin of biological samples, from which the 25 ANV capsid sequences were obtained, are summarised in Table 1. Eighteen samples were collected from flocks belonging to 5 UK poultry organisations during 2004-2008, with the majority being obtained from a single organisation (organisation E). Nine sequences were obtained from samples that were collected in longitudinal surveys of 4 broiler flocks at early (day 4/ 5) and late (day 35) time points in the flocks' life.

Pairwise comparisons of ANV capsid sequences. The nucleotide and deduced amino acid sequences of the ANV capsid genes were identified by sequence analysis of the 2.5kbp fragment sequences. Comparison of the 27 capsid nucleotide sequences,

including those of ANV-1 and ANV-2, showed that the capsid genes ranged in size from 2007 to 2049 nucleotides and encoded proteins ranging from 669 to 683 amino acids. Pairwise comparisons showed that nucleotide identities ranged from 59 to 100%. Similar amino acid comparisons showed that the pairwise amino acid identity values ranged from 54 to 100%, with the most distantly related sequences being those of VF08-29a and VF08-16. From the pairwise amino acid comparisons, the capsid sequences of ANV-1, ANV-2, VF04-1/2, VF07-13/7, VF08-3a and VF08-65 were selected to be representative of 6 different groups. These shared less than 80% amino acid identity with one another (Table 2), with only 4 of the 15 pairwise comparisons sharing amino acid identities greater than 70%. VF08-65 shared the lowest amino acid identity values (55-57%) with the other 5 representative ANVs. These low levels were still substantially greater than those shared by the 6 ANVs with any of the four other avian astroviruses, turkey astrovirus types 1 (TAsV-1) and 2 (TAsV-2), chicken astrovirus (CAstV) and duck astrovirus (DAstV) (Table 2). It was noted that the 6 ANVs shared higher identities with TAsV-1 (38-41%) than with CAstV (28-29%), TAsV-2 (24-27%) and DAstV (23-27%).

Each of the remaining 21 capsid sequences shared greater than 80% amino acid identity with at least one of the 6 representative ANVs, and each was then grouped with the representative ANV, with which it shared highest amino acid identity (Table 3). For example, 06V0062, VF08-29a and VF08-29b were grouped with ANV-1, with which they shared an intra-group identity range of 82-94%. Other intra-group amino acid identities were 82-94% (ANV-2 – like), 87-100% (VF04-1/2 – like), 79-99% (VF07-13/7 – like) and 80-95% (VF08-3a – like). None of the 21 capsid sequences resembled VF08-65, the only member of the sixth group. Comparison of the inter-group identities showed that the most closely related groups were the ANV-1 – like and ANV-2 – like groups, which displayed an amino acid identity range of 70-82%, while the VF08-65 sequence displayed the lowest identities (range 55-58%) with the sequences from the other groups.

Phylogenetic analysis of complete ANV capsid sequences. The 27 ANV capsid amino acid sequences were compared phylogenetically using the neighbour-joining method (Figure 1). Deduced capsid sequences of other avian astroviruses (TAsV-1, TAsV-2, CAstV and DAstV) and the more distantly-related human astrovirus (HAstV) were also included, and these grouped separately from the ANV capsid

sequences. Close examination showed that the phylogenetic distribution of the ANV sequences shared similarities with the groupings derived from the pairwise comparisons (Table 2). Two major clusters were observed. One of these comprised 2 sub-clusters, which separately corresponded to the ANV-1- like and ANV-2- like groups. The other major cluster also comprised two sub-clusters, one of which corresponded to the VF04-1/2 – like group, while the other contained the VF07-13/7 – like and the VF08-3a – like groups. The VF08-65 ANV represented the most distantly-related sequence, separated from the 2 major clusters.

Examination of the pairwise comparison (Table 2) and phylogenetic analysis (Figure 1) provided little evidence that the ANV sequences had been assorted on the basis of geographical location or year of sampling (Table 1). For example, ANVs closely similar to ANV-1, which was isolated in Japan in the late 1970s, were detected in Germany in 2006 and in the UK in 2008, while ANV-2 – like viruses were detected in Japan in the late 1970s and in the UK in 1983 and 2008. From a geographical standpoint, the pairs of ANV sequences from Japan (ANV-1, ANV-2) and Germany (05V0105, 06V0062) were more closely related to UK viruses than to each other. The results also indicated that viruses representing several different ANVs groups may be in circulation within a relatively small geographic location and within a relatively short timeframe. This was evident when 11 samples collected in 2008 from the same UK poultry organisation were found to contain ANVs that could be assigned to 5 of the 6 groups. Further analysis of longitudinal survey samples taken at day 4 or day 5 and at day 35 from 4 broiler flocks showed that in 2 flocks, the ANVs detected at different time points belonged to different groups, for example VF08-29a and VF08-60, which were ANV-1 –like and ANV-2 –like respectively. Although the other 2 pairs of samples were VF07-13/7 –like, the viruses collected at the early and late time points differed with regard to their capsid sequences. For example VF08-21 and VF08-52 shared 91% amino acid identity, while VF08-22 and VF08-53 shared 81% identity.

The detection of sequence-distinct capsids in clones produced from samples obtained from small pools of birds collected at the same time point (VF08-3a/b, VF08-29a/b) provided a further indication of the diverse nature of the ANVs in circulation. It was noted that one of the 6 ANV groups, (VF04-1/2 –like), was not detected in the 14 samples investigated from 2008. This group comprised 5 ANVs, which were detected in 2004-2006 in samples sourced from four UK poultry

organisations, severely affected by outbreaks of “uneven growth”, and from one German poultry organisation, sampled in 2005. From a disease standpoint, it was also noted that the Belgium ELV1 and EF91-276Cl3 capsid sequences, which were derived from severe runting-stunting cases that affected European poultry organisations in the 1980s, were closely related (94% amino acid identity).

Identification of variable regions. The amino acid sequences of the 27 ANV capsids were analysed by Clustal W alignment. Initial comparison of the 6 representative ANVs showed the presence of relatively short (up to 7 amino acids) conserved or semi-conserved regions, and a number of variable regions, ranging from approximately 15 to approximately 30 amino acids in size (Figure 2). Nine of these variable regions, located throughout the capsid sequence, were selected to facilitate more detailed comparisons of the 27 ANVs. Using the amino acid positions of the reference ANV-1 strain, the 9 selected variable regions were located at residues 15-38 (A), residues 113-127 (B), residues 221-240 (C), residues 338-355 (D), residues 393-419 (E), residues 433-454 (F), residues 460-488 (G), residues 520-544 (H) and residues 615-641 (I). When corresponding variable regions from the 6 representative ANV sequences were compared, these regions differed greatly in their amino acid variability, with the most variable being regions C (mean identity 37.1%; range 5-81%), E (mean identity 42.5%; range 23-65%) and I (mean identity 28.1%; range 5-96%). Corresponding variable regions also differed with regard to the number of amino acids present, with these sequences frequently being the sites for deletions (Figure 2). For the purposes of this investigation, we considered variable regions from two ANV capsids to be similar if there were amino acid changes in less than 25% of the residues present. Using this criterion, pairwise alignment comparisons showed that the majority of the 9 variable regions present in the 6 representative ANVs were specific to the representative virus (Table 4). There were two major exceptions. Thus, variable regions F, G, H and I of ANV-2 resembled their counterparts in VF04-1/2, the third representative ANV, and variable regions A, B, C and D of representative ANVs VF07-13/7 and VF08-3a were considered to be similar.

The remaining 21 ANVs were then analysed using the same criterion to determine how their variable region sequences compared with those of the 6 representative ANVs (Table 4). Only 5 (VF05-1/8, VF06-1/1, VF06-1/4, VF08-18/16 and VF08-18/14) of the 21 were found to be indistinguishable from any of the 6

representative ANVs in terms of their variable region profiles. Other ANV capsids displayed profiles, in which most but not all of the variable regions resembled those of a representative ANV. For example, in both EF91-276C15 and VF08-39, 8 of the 9 variable regions were similar to those of ANV-2, while VF08-29b had a profile, in which 7 of the 9 variable regions were similar to those of ANV-1. The majority of the 21 ANV capsid sequences displayed variable region profiles that differed substantially from those of the 6 representative ANVs. Some of these such as 06V0062, VF08-22, VF08-21, VF08-18/5 and VF08-53 contained distinctive sequences in at least 3 of the 9 variable regions, while others displayed profiles in which the variable regions from different representative ANVs were found in novel arrangements. For example, in VF08-60, EF91-276C13 and BelgELV1, variable regions A - E, which were characteristic of the N-terminal region of ANV-2, were found in combination with variable regions F, G and H, which were characteristic of the C-terminal region of ANV-1. C-terminal capsid sequences containing variable regions F to I, which were characteristic of ANV-1, were also found in combination with N-terminal variable regions A to E, which were characteristic of the third representative ANV, VF04-1/2 (VF05-1/5 and 05V0105), and the fourth representative ANV, VF07-13/7 (VF08-3b). Conversely, VF08-29a displayed N-terminal variable regions that were largely characteristic of ANV-1 and C-terminal variable regions F to I that corresponded to those of ANV-2.

Overall, when the 27 ANVs were compared in this way, it was noted that some of the variable regions displayed greater variability than others. For example, with variable regions A - E, G and H, 5-7 different sequences were recognised, whereas 12 and 11 different sequences were identified for variable regions F and I respectively.

Phylogenetic and pairwise comparison of ANV C-terminal 427-end regions.

Examination of the data presented in Table 4 indicated that 9 and 8 of the 27 ANVs had variable regions F to I that resembled those of ANV-1 and ANV-2 respectively. On the basis that a substantial C-terminal region of the astrovirus capsid is likely to be located on the outside of the capsid and, as such, to have increased importance in relation to antigenic diversity (see Discussion), capsid sequences comprising amino acid residues 427-end were identified for each of the 27 ANV capsids and used for phylogenetic analysis and pairwise comparison. The phylogenetic tree based on these

C-terminal capsid regions comprised 2 major clusters and a single outlying ANV, VF08-65, labelled "F" (Figure 3). One of the major clusters comprised 3 sub-clusters, which we have labelled groups A, B and D in accordance with the pairwise comparison findings (Table 5). Groups A and B contained ANVs with C-terminal regions that are closely related to those of ANV-1 and ANV-2/ VF04-1/2 respectively, while group D contained VF08-3a and VF08-18/14. The other major cluster comprised groups/ virus that we have labelled C and E. Group C contains ANVs with C-terminal regions that are closely related to that of VF07-13/7, while the outlying ANV, VF08-53, within this cluster was labelled "E". The 27 ANV C-terminal regions were analysed by pairwise comparison using the 6 groups, A-F (Table 5). Intra-group identities were 80% or greater, while the highest inter-group identities occurred between groups A and B (68-72%), which contained the ANVs with C-terminal sequences resembling those of ANV-1 and ANV-2 respectively and between groups C (ANVs with C-terminal regions resembling those of VF07-13/7) and E (VF08-53) with an inter-group identity of 70-73%.

Evidence for recombination. Examination of the variable region profiles shown in Table 5 and the phylogenetic trees based on the complete capsid and C-terminal capsid regions (Figures 2, 4) supported the view that some of the ANV capsid sequences may have been derived from recombination events in which N-terminal capsid regions specified by one ANV were combined with C-terminal capsid regions specified by a different ANV. Analysis of VF08-60, EF91-276C13, BelgELV1, VF05-1/5, 05V0105 and VF08-3b sequences, all of which contained C-terminal regions resembling that of ANV-1, indicated that the site for possible recombination was located between variable regions E and F.

The possibility that viral RNA recombination events were responsible for the varying combinations of N- and C-terminal capsid sequences was investigated by applying the SimPlot computer programme. The examples chosen for investigation were EF91-276C13, VF05-1/5 and VF08-3b, all of which contained C-terminal regions similar to that of ANV-1, in combination with N-terminal regions that were characteristic of representative viruses ANV-2, VF04-1/2 and VF08-3a respectively. Using SimPlot analysis, the capsid sequence of VF08-3b was compared with that of ANV-1 and VF08-3a, and the result showed that the identity values shared by VF08-3b with VF08-3a were high for the first 1200-1250 nucleotides, whereas the identities

shared with ANV-1 were very low. From nucleotide 1250-1300, the reverse situation was observed, with high identity values now being shared with ANV-1 and low levels being shared with VF08-3a (Figure 4). The recombination site was estimated to be approximately 1275 nucleotides. Additional SimPlots were undertaken with the capsid sequence of EF91-276Cl3, which was compared with the sequences of ANV-1 and ANV-2, and the sequence of VF05-1/5, which was compared to those of ANV-1 and VF04-1/2. These produced similar plots, from which recombination sites of 1325 and 1290 were estimated. The estimated recombination sites of nucleotides 1275, 1325 and 1290 corresponded to amino acid residues of 425, 442 and 430 respectively.

Discussion

This is the first paper describing sequence variation of complete ANV capsid protein genes. Earlier studies involving relatively small numbers of ANVs, reported levels of amino acid sequence identity of 88-93% for partial ORF 1a sequences (Mandoki *et al.*, 2006a) and 92-100% for the partial ORF 1b sequences (Pantin-Jackwood *et al.*, 2006a). These levels are substantially lower than those determined in our comparison of 27 ANV capsid protein gene sequences where pairwise amino acid identities as low as 51% were determined. On the basis that the astrovirus capsid protein interacts directly with host cell receptors and with the host's immune system, the high level of diversity that we have observed is likely to have important implications for virus tropism, pathogenesis and immune protection.

Pairwise comparisons supported by phylogenetic analysis allowed the identification of 6 ANV capsid sequences, including those of the previously known serotype 1 (ANV-1) and serotype 2 (ANV-2) strains, which were selected to be representative of 6 ANV groups (Tables 2, 3). The most distantly related groups were the ANV-1-like and VF07-13/7-like groups, which shared an amino acid identity range of 54-58%, while the ANV-1-like and ANV-2-like groups were the most closely related groups, sharing 70-82% amino acid identity. Intra-group amino acid identity values were 80% or greater. Although the levels of antigenic diversity cannot be predicted accurately from pairwise amino acid identity values, based on findings with human and turkey astrovirus serotypes, it is probable that the VF04-1/2, VF07-13/7, VF08-3a and VF08-65 ANVs will be antigenically very different from ANV-1

and ANV-2, and from each other. Pairwise amino acid comparisons showed that 8 different HAstV serotypes shared a mean identity of 68.3%, with the most distantly related serotypes, 5 and 7, sharing 58.6 % identity, while a relatively high 84.3% identity value was shared by serotypes 3 and 7 (Tang *et al.*, 2005). A similarly high amino acid identity, 82.8%, was shared by two different TAstV-2 serotypes. Pairwise amino acid comparisons of the 6 representative ANVs (Table 2), displayed a mean identity of 62.4%, with the most closely related representatives, VF07-13/7 and VF08-3a, sharing 78% identity. These results suggested that the 6 representative ANVs are likely to belong to different serotypes.

However, pairwise comparison of the amino acid identities shared by complete capsid proteins is likely to be a very crude tool for predicting ANV serotypic differences, and it may prove more useful to focus on the sequence variation exhibited by the C-terminal capsid regions. Based on capsid protein analyses of human and animal astroviruses, including ANV and TAstV, Krishna (2005) predicted that the astrovirus capsid protein comprises 2 structural domains, with the N-terminal 1-415 amino acid residues having virion assembly and RNA-packaging functions, and the C-terminal 416-end residues having the determinants required for receptor-binding and internalisation. The identification of neutralising epitopes in the HAstV capsid proteins that were encoded by the C-terminal half of the capsid protein is also consistent with the C-terminal 416-end region having a surface-orientated location (Sanchez- Fauquier *et al.*, 1994; Bass & Upadhyapuda, 1997). Therefore, differences in the C-terminal half of the ANV capsid are likely to be more important in relation to antigenic diversity, including serotypic variation, than the N-terminal half. When the C-terminal 427-end regions of 27 ANV capsids were analysed phylogenetically and by pairwise comparison, we found evidence for three major groupings, A-C, and 2 minor groupings, D and E. The major groupings were (A) ANVs with C-terminal regions similar to ANV-1, (B) ANVs those with C-terminal regions similar to ANV-2 and VF04-1/2, and (C) ANVs with C-terminal regions similar to VF07-13/7. It remains to be determined whether viruses which share similar C-terminal halves are more closely related in terms of their cross-immunofluorescence and cross-virus neutralization reactivities than those that share similar N-terminal halves. The sequence diversities within these three C-terminal groupings are substantial (Table 5), and it is reasonable to assume that serotypic differences not only exist between viruses from the 3 different groups but also between viruses belonging to the same

group. Although, at present the precise location(s) of the ANV neutralising epitope(s) remain(s) unknown, it is likely that it/ they will correspond to one or more of the C-terminal variable regions F-I, which allowed the serotypically different ANV-1 and ANV-2 strains to be distinguished.

The different phylogenetic distributions of the 27 ANVs, which resulted following comparisons of the complete capsid sequences (Figure 1) and C-terminal 427-end sequences (Figure 3) provided strong indications that viral RNA recombination events had taken place. Subsequent SimPlot analyses (Figure 4) showed that this was the case for VF08-3b, VF05-1/5 and EF276C13, all of which had C-terminal regions that resembled that of ANV-1 in combination with different N-terminal regions. For these 3 ANVs, approximate recombination sites were estimated at nucleotide positions 1275, 1290 and 1325, suggesting that this region may be a hot-spot for recombination. Recombination by avian astrovirus RNAs has been shown previously for TAsTV (Pantin-Jackwood *et al.*, 2006b; Strain *et al.*, 2008), where sites between ORF1b and ORF2 and within ORF 2 were identified, and recombination is likely to provide an important mechanism by which ANV sequence diversity can be increased.

The substantial capsid sequence diversity that we have described is particularly noteworthy given the relatively small number of sequences compared and the relatively small number of different sources from which the samples were obtained. For example, ANVs from 5 of the 6 representative groups were detected in 11 samples that were collected from 6 flocks belonging to the same UK poultry over a 4-month period in 2008. Furthermore, ANVs from different representative groups were detected in the same broiler flocks sampled at different times (VF08-29a and VF08-65) and from different birds sampled from the same flock at the same time (VF08-18/14 and VF08/16). Together these findings support the view that infections with genetically distinct ANVs are widespread and that infections of the same broiler flock with more than one genetic variant are not uncommon. The co-circulation of ANVs, which are likely to exhibit substantial antigenic differences (including serotypic differences), has important implications for several aspects of commercial poultry production. Work undertaken in Japan showed that ANVs belonging to different serotypes shared very low levels of cross-reactivity by IIF (Shirai *et al.*, 1992), and this has led us to suggest that serological tests, including IIF or ELISA, which use antigen specified by one ANV serotype, such as the commonly used G4260

strain (ANV-1), are unlikely to be efficient in detecting antibodies induced by serotypically different ANVs. As a consequence, SPF poultry producing organisations, which use serological testing to demonstrate freedom from ANV infection, may be failing to detect infections with ANVs that are antigenically different from the isolate on which their antibody-detecting test is based. Given that ANV can be vertically transmitted (Todd *et al.*, unpublished results), undetected ANV infections within SPF breeding flocks could lead potentially to ANV contamination of avian vaccines that are produced in chick embryos. The common occurrence of geneically and antigenically different ANVs has also important implications if vaccine-based control strategies of ANV-associated disease problems are being considered. Vaccination of breeders during rear has the potential to prevent vertical transmission and to ensure uniformly high levels of maternal antibody, which are likely to reduce the severity of early virus infections of broiler chicks. It is known that ANV infections will cause less pathogenic effects in embryos or young chicks derived from immunised hens (Takase *et al.*, 1994). A vaccine-based approach will have to take into consideration the widespread and high prevalence of antigenically diverse ANVs. This might involve producing composite vaccines utilising antigens specific to selected ANV strains. Since submission of this paper, Pantin-Jackwood *et al.* (2010) have recently described the sequence variation displayed by partial capsid sequences of 9 ANVs that originated in the USA.

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Figure Legends

Figure 1. *Phylogenetic tree of ANVs, other avian astroviruses and human astrovirus (HAstV) based on capsid amino acid sequences. The tree was constructed using Mega 4 (Tamura et al., 2007) using the neighbour-joining method and 1000 bootstrap replicates (bootstrap values are shown on the tree). Data relating to the origin of the ANVs including the Genbank accession numbers are shown in Table 1. The Genbank accession numbers of the other avian and human astroviruses are Y15936 (TAsV-1), HQ330506 (CAstV), NC_005790 (TAsV-1), FJ434664 (DAstV) and AAC60723 (HAstV serotype 1).*

Figure 2. *ClustalW alignment of C-terminal amino acid (residues 427-end) regions of the 6 representative ANVs. Variable regions F, G, H and I are shown in shaded*

boxes. Asterisks denote amino acids shared by all six representative ANVs, while full stops and colons denote amino acids with shared characteristics.

Figure 3. Phylogenetic tree of ANVs based on C-terminal (residues 427-end) region amino acid sequences. The tree was constructed using Mega 4 (Tamura et al., 2007) using the neighbour-joining method and 1000 bootstrap replicates (bootstrap values are shown on the tree). Data relating to the origin of the ANVs including the Genbank accession numbers are shown in Table 1.

Figure 4. SimPlot analysis in which the capsid gene nucleotide sequence of VF08-3b (Query) was compared with those of ANV-1 and VF08-3a. The capsid genes were compared by sequence alignment using a window of 200 nucleotides, which was moved along in 20-nucleotide steps (Lole et al., 1999). Data relating to the origin of the ANVs including the Genbank accession numbers are shown in Table 1.

Table 1. *Origin of avian nephritis virus capsid sequences used in this study.*

Identifier	Country/ Organisation ^a / house ^b / age ^c	Year	Accession number
ANV-1	Japan	1979	NC_003790
ANV-2	Japan	unknown	AB046864
EF91-276C15	UK	1984	HQ330484
EF91-276C13	UK	1984	HQ330485
BelgELV1	Belgium	1980s	HQ330486
05V0105	Germany	2005	HQ330497
06V0062	Germany	2006	HQ330496
VF04-1/2	UK/ C/a/10-24day	2004	HQ330481
VF05-1/5	UK/ G/a/8day	2005	HQ330487
VF05-1/8	UK/ G/a/4day	2005	HQ330493
VF06-1/1	UK/ A/a/7day	2006	HQ330494
VF06-1/4	UK/ A/d/14day	2006	HQ330495
VF07-13/7	UK/ A/h/ 14day	2007	HQ330482
VF08-3a	UK/ D/a/22day	2008	HQ330483
VF08-3b	UK/ D/a/22day	2008	HQ330488
VF08-18/5	UK/ E/b/35day	2008	HQ330490
VF08-18/14	UK/ E/c/35day	2008	HQ330489
VF08-18/16	UK/ E/c/35day	2008	HQ330498
VF08-21	UK/ E/d/5day	2008	HQ330501
VF08-22	UK/ E/e/5day	2008	HQ330500
VF08-29a	UK/ E/f/4day	2008	HQ330491
VF08-29b	UK/ E/f/4day	2008	HQ330492
VF08-39	UK/ E/g/4day	2008	HQ330503
VF08-52	UK/ E/d/35day	2008	HQ330502
VF08-53	UK/ E/e/35day	2008	HQ330499
VF08-60	UK/ E/f/35day	2008	HQ330505
VF08-65	UK/ E/g/35day	2008	HQ330504

^a Upper case letters denote different poultry organisations.

^b Lower case letters denote different farms/ flocks poultry houses.

^c Age in days where known.

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Table 2. *Pairwise comparison of amino acid identities shared by the 6 representative avian nephritis viruses and other avian astroviruses.*

	ANV-1	ANV-2	VF04-1/2	VF07-13/7	VF08-3a	VF08-65	TAstV-1 ^a	CAstV ^b	TAstV-2 ^c	DAstV ^d
ANV-1	-	71	68	60	64	57	41	28	26	24
ANV-2		-	73	58	61	56	39	27	26	23
VF04-1/2			-	68	73	57	40	28	27	25
VF07-13/7				-	79	58	42	28	28	27
VF08-3a					-	58	41	28	28	27
VF08-65						-	39	29	27	26
TAstV-1 ^a							-	35	36	36
CAstV ^b								-	34	33
TAstV-2 ^c									-	70
DAstV ^d										-

^{a, b, c and d} The capsid sequences of other avian astroviruses were derived from sequences available in Genbank databases (See legend to Figure. 1 for Accession numbers).

Table 3. Pairwise comparison of amino acid identities shared by the 6 avian nephritis virus groups

	1. ANV-1 – like	2. ANV-2 – like	3. VF04-1/2 – like	4. VF07-13/7 – like	5. VF08-3a – like	6. VF08-65
1. ANV-1 – like (06V0062, VF08-29a, VF08-29b)	82-94	70-82	64-73	54-58	61-73	56-57
2. ANV-2 – like (EF276C15, VF08-39, VF08-60, EF276C13, BelgELV1)		82-94	64-74	55-58	60-72	56-58
3. VF04-1/2 – like (VF06-1/1, VF05-1/8, VF06-1/4, 05V0105, VF05-1/5)			87-100	64-71	69-82	55
4. VF07-13/7 – like (VF08-18/16, VF08-52, VF08-22, VF08-21, VF08-53, VF08-18/5)				79-99	70-80	56
5. VF08-3a – like (VF08-18/14, VF08-3b)					80-95	55-56
6. VF08-65 ^a						100

^a VF08-65 is the only member of the sixth group.

Table 4. Comparative analyses of avian nephritis virus capsids in terms of the similarities shared by variable region sequences (A-I)

	A (15-38) ^a	B (113-127)	C (221-240)	D (338-355)	E (393-419)	F (433-454)	G (460-488)	H (520-544)	I (615-641)
ANV-1	1 ^b	1	1	1	1	1	1	1	1
ANV-2	2	2	2	2	2	2/3	2/3	2/3	2/3
VF04-1/2	3/4/5	3	3	3	3	2/3	2/3	2/3	2/3
VF07-13/7	3/4/5	4/5	4/5	4/5	4	4	4	4	4
VF08-3a	3/4/5	4/5	4/5	4/5	5	5	5	5	5
VF08-65	6	6	6	6	6	6	6	6	6
06V0062	06V0062 ^c	1	1	1	06V0062/ 08-29b	1	1	1	06V0062
VF08-29b	1	1	1	1	08-29b/ 06V0062	1	1	1	08-29b
VF08-29a	08-29a	1	1	1	08-29a	2/3	2/3	2/3	2/3
EF276C15	2	2	276C15/ 08-39 ^d	2	2	2/3	2/3	2/3	2/3
VF08-39	2	2	276C15/ 08-39	2	2	2/3	2/3	2/3	2/3
VF08-60	2	2	6	2	2	1	1	1	08-60
EF276C13	2	2	6	2	2	1	1	1	276C13
BelgELV1	2	2	6	2	BelgELV1	1	1	1	BelgELV1
VF06-1/1	3/4/5	3	3	3	3	2/3	2/3	2/3	2/3
VF06-1/4	3/4/5	3	3	3	3	2/3	2/3	2/3	2/3
VF05-1/8	3/4/5	3	3	3	3	2/3	2/3	2/3	2/3
VF05-1/5	3/4/5	3	3	3	3	1	1	1	1
05V0105	3/4/5	3	3	3	3	1	1	1	1
VF08-18/16	3/4/5	4/5	4/5	4/5	4	4	4	4	4
VF08-52	3/4/5	4/5	4/5	4/5	4	08-52/ 08-22/ 08-21	4	4	4
VF08-22	3/4/5	4/5	08-22/ 08-21/ 08-18/5	3	08-22/ 08-21/ 08-18/5	08-52/ 08-22/ 08-21	4	4	4
VF08-21	3/4/5	4/5	08-22/ 08-21/ 08-18/5	3	08-22/ 08-21/ 08-18/5	08-52/ 08-22/ 08-21	4	4	4
VF08-18/5	3/4/5	4/5	08-22/ 08-21/ 08-18/5	3	08-18/5 / 08-22/ 08-21	08-18/5	4	4	4
VF08-53	3/4/5	4/5	4/5	4/5	08-53	4	08-53	08-53	08-53
VF08-3b	3/4/5	4/5	4/5	4/5	08-3b	1	1/ 2	1	1
VF08-18/14	3/4/5	4/5	4/5	4/5	5	5	5	5	5
Total ^e	6	5	7	5	12	7	6	6	11

^a numbers in parentheses are those of amino acid residues of variable region using the ANV-1 strain as reference.

^b numbers relate to the representative ANV with which the variable region sequence of the test ANV shares greater than 75% amino acid identity.

^c variable region shares 75% or less amino acid identity with any of 6 representative ANVs.

^d variable regions of 2 or more ANVs differ from those of 6 representative ANVs but share greater than 75% identity with one another.

^e number of different sequences recognised following comparison of 27 ANVs.

Table 5. Pairwise amino acid identity comparison of avian nephritis virus groups A-E, grouped according to their C-terminal 427-end capsid region identities

	A	B	C	D	E (VF08-53)	F (VF08-65)
A. ANV-1, VF08-3b, 05V0105, VF05-1/5, 06V0062, BelgELV1, EF276C13, VF08-29b, VF08-60	86-98	68-72	52-54	61-64	51-54	48-50
B. ANV-2, EF276C15, VF08-29a, VF08-39, VF06-1/4, VF06-1/1, VF04-1/2, VF05-1/8		88-100	50-52	61-64	52-54	47-48
C. VF07-13/7, VF08-21, VF08-22, VF08-52, VF08-18/5, VF08-16			91-100	51-56	70-73	49-50
D. VF08-3a, VF08-18/14				80-98	57-60	51-54
E. VF08-53 ^a					100	52
F. VF08-65 ^a						100

^a VF08-53 and VF08-65 are the only members of groups E and F respectively.

Fig 1

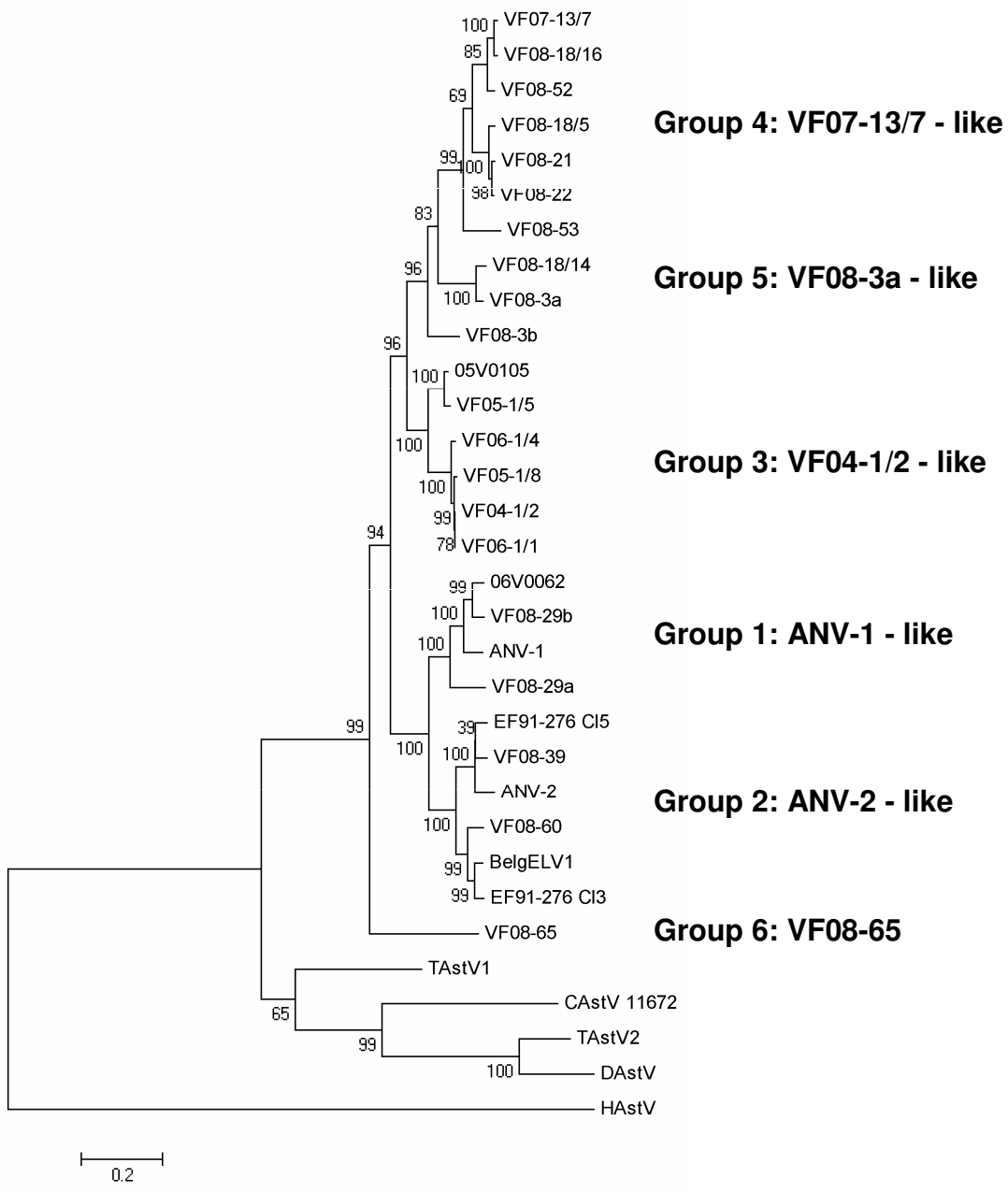


Fig. 3

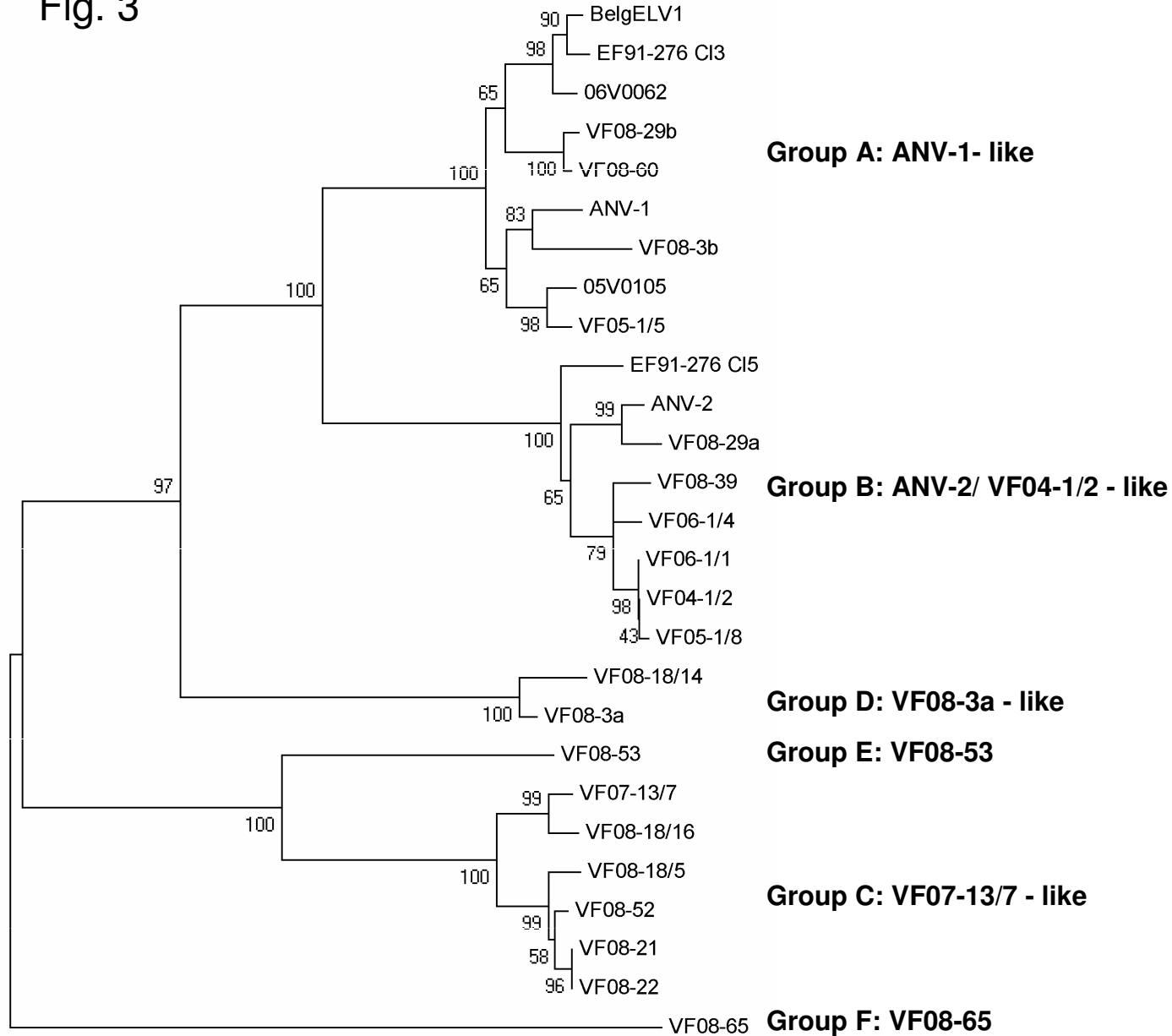


Fig. 4

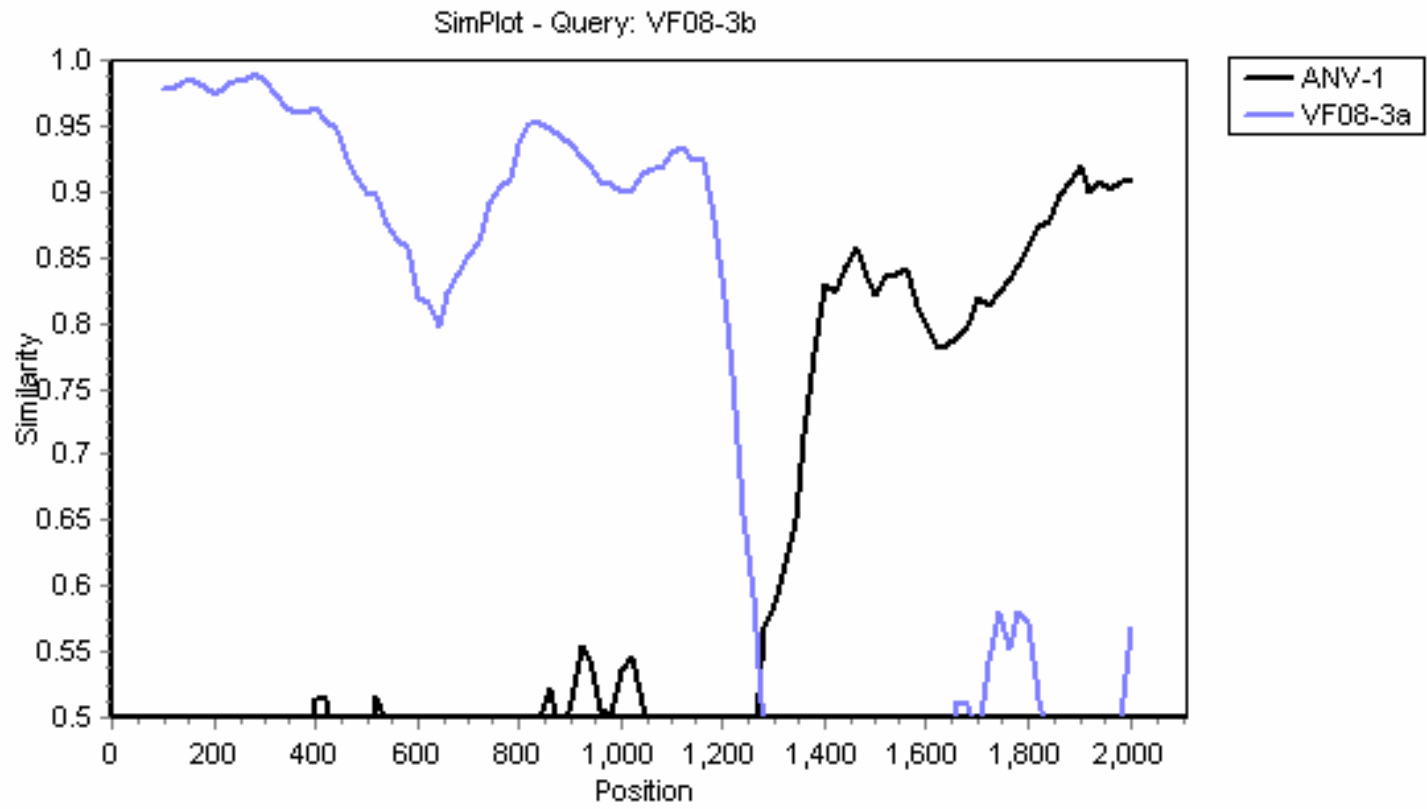


Fig. 2

	Variable region F	Variable region G	
ANV-2	GQDGV PANYTFNG--DSYTAQADWRG STLVLTGIPKHKRVAGNLSNFGVVTNQ---MSKV		55
VF04-1/2	GQDGV PANYTFNG--DSYTGQADWRG STLVLTGIPKHKRVAGSLANFGVVTNQ---MSKV		55
ANV-1	GQPGV PLYTFDG--SSYTPPTNWL GSTILLTGIPAHKRV TGNLAKFGVTNLQ ---MSKV		55
VF08-3a	GQAGV PAVYEYTG--DAYTPQPRWT GSTIFLTGVPYHTRATGATQSF GVRTNN ---MSPS		55
VF07-13/7	GQSGV PALYTFNSGTGAYTPMTRWT GSTLLLTGVPYELRSGSSQ QFGVRVQNSPGLSPA		60
VF08-65	GQRGV PLYTYSG--GSYEVSPNWL GSTMLLTGLPRYRRVIGGSQ QFGPNPNG ---MQPF		55
	** *** . * : . . : * . * * . : . * * * : * : . * . * * .		
		Variable region H	
ANV-2	TTTAL E IYDF TDFGIVF GGGYQLQEGGIHTGKTLVHSLMTGAPIK P WLYATQSSTTWYWP		115
VF04-1/2	TTTAL E IYDF TDFGI FFGGGYQLQEGGVHTGKTMVHSLMTGAPIK P WLYATQSSTTWYWP		115
ANV-1	AATA L EIYDF TDFGV VFFGTGSYLSEGGIHTGKTLIYSLMSGQTPNPWLAANQSGTTWYMP		115
VF08-3a	NCTTLD I YDF TDFGV VFFGSNGYLSQGA I H T SKTMIYSLKTNPNINPWLAANQSSTTWSMP		115
VF07-13/7	AATS I QIYDF TKFGI FFGAGEFLGQGGVHTAKTLLTAITASSNP-PWLD C SR--YT S WSP		117
VF08-65	ACD S LDVYDF TDNGV FIGFGTYQNGAIHTGKTL L YSIKNSPDRKPWLGAEK--YT W QWP		113
	: : : : * * * * . * : : : * . : * : : * * . * * : : : . * * * . : * * *		
ANV-2	DWT---GFPK P GE G DYFLQMQD T DR T HT T TCVGIYIVVAYHQ S RR L IAFF N NA-GPVRA		171
VF04-1/2	TWT---GFPQ P GE G DYFLQMQD T DR T HT T CVSVYLLVAYRAS R RL I AF Y NNG-GPVRA		171
ANV-1	SWA---GFPQ P GG D YFLQMQD V DT T HT T SVNVYFLVAYR Q SR L IAFF N TG-GTARP		171
VF08-3a	TWS---GYP A PG Q GDYFLQMQD T DT T HT T SVGCYFLVMYGES R KL V AF F NTGT G TARP		172
VF07-13/7	DWLT S AGY P K P A Q GD W WLQMQK V GD T SH T TPVGIYFLIAYEEM Q L V AF W HTG-SGAQA		176
VF08-65	TWV V Q Q GY P Q K Q G D W WLQ T Q N T T DT S HT V ATRCYFLVAYR D E Q KL V AF W NNN-TGSQA		172
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		Variable region I	
ANV-2	APT T MLC L YNVDAGR A P A TPYNT F QLTLQSE---G T DP N SP S E D ED D DD D ISLAGSCLQDEF		228
VF04-1/2	APT T MLC L YNVDAGR A P A TPYNT F QLTLQSE---G A DP N SP S E D ED D DD D ISLAGSCLQDEF		228
ANV-1	APT S MLC L YNVDCGR A P Q TPYPT F QSTLQSLNQIGVDAKS-DP D S D DD D ISLAGSVIGDEF		230
VF08-3a	ALSS M MLC L YNVDAGR A P V RI Q G F LL S PS Q NF----V E T D N Q D N DD D DD D ISLAGSCLQDEF		228
VF07-13/7	EPT S LLC L YNVDAGR A P V RV P H F I I TT T ARN---E V E V D G -G D S D DD D ISLAGSCV G DEF		232
VF08-65	AP S T I M C L Y NVDAGR A P V LT S G H L P P F IL P SN--E V E V D G ----D D DD D ISLAD S CL R E E F		226
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ANV-2	DCVD Q LE K ER E DL M RR L R D LD L RR F QI		255
VF04-1/2	DCVD Q LE K ER E DL M RR L R D LD L RR F QI		255
ANV-1	DS V D H LE R ER E DL M RR L R D LD L RR F QI		257
VF08-3a	DCVD Q LE K ER E DL M RR L R D LD L RR F QI		255
VF07-13/7	EG V D Q LE R ER A EL M S R LR D LD L RR F QI		259
VF08-65	DC V E T Y E Q E R D R L I K RL R E L D F H R F Q V		253
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