Title

Temporally separated feline calicivirus isolates do not cluster phylogenetically and are similarly neutralised by vaccine strain FCV-F9 anti-sera *in vitro*

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Abstract

Objectives

Feline calicivirus is a highly variable and globally important feline pathogen for which vaccination has been the mainstay of control. Here we test whether the continued use of FCV-F9, one of the most frequently used vaccine strains globally, is driving the emergence of vaccine-resistant viruses in the field.

Methods

This study made use of two representative panels of field isolates previously collected from cats visiting randomly selected veterinary practices across the UK as part of separate cross-sectional studies from 2001 and 2013/2014. Phylogenetic analysis and *in vitro* virus neutralisation tests were used to compare the genetic and antigenic relationships between these populations and FCV-F9.

Results

Phylogenetic analysis showed a typically radial distribution dominated by 52 distinct strains, with strains from both 2001 and 2013/2014 intermingled. The sequence for FCV-F9 appeared integral to this phylogeny and there were no significant differences in the genetic distances within each studied population (intra-population distances), or between them (inter-population distances) or between each population and FCV-F9. A 1 in 8 dilution neutralised 97 and 100 % of the 2001 and 2013/14 isolates respectively and a 1 in 16 dilution neutralised 87 and 75% of isolates respectively. There was no significant difference either in variance between the FCV-F9 neutralising titres for the two populations or for the distribution of neutralisation titres across the two populations.

Conclusion and relevance

Although FCV is a highly variable virus, we find no evidence for a progressive divergence of field virus from vaccine strain FCV-F9 either phylogenetically or antigenically, with FCV-F9 antisera remaining broadly and equally cross-reactive to two geographically representative and temporally separated FCV populations. This suggests that the immuno-dominant region of the FCV capsid responsible for neutralisation may have structural constraints which prevent its longer-term progressive antigenic evolution.

Introduction

Feline calicivirus (FCV) is a common pathogen of cats inducing acute oral and upper respiratory tract disease (URTD) 1. As an RNA virus, its genome is often inaccurately replicated leading to high sequence variability and antigenic variation 2-8. Most commercially available live FCV vaccines are based on FCV-F9 9, 10, a strain isolated in 1958 11 and selected for its broad *in vitro* cross-reactivity 8. It has been suggested the continued use of FCV-F9 in this variable genetic background may be driving the emergence of vaccine-resistant strains and that the efficacy of such vaccines may be reducing over time 12-14.

We recently reported that FCV-F9 remains broadly cross-reactive to representative FCV isolates collected across Europe between 2013 and 2014 15. Comparison of the data from this study with results from a previous study performed 12 years earlier 16 suggested that FCV-F9 anti-serum neutralised the contemporary isolates as effectively as the earlier isolates. However, although the two studies were performed in a similar manner, the FCV-F9 anti-sera used for the neutralisation tests were raised in different cats and using different methodologies (infection vs vaccine overdose), making direct comparison of results difficult. Here we test the hypothesis that field isolates of FCV are evolving increased resistance to FCV-F9 vaccines by directly comparing the *in vitro* cross-reactivity of a single FCV-F9 anti-serum with representative UK FCV isolates collected in 2001 and 2013/14, together with phylogenetic comparisons of the viruses in our sample populations.

Materials and Methods

Viruses

Field isolates were previously collected from veterinary practices recruited randomly across the UK as part of two cross-sectional studies in 2001 16 and 2013/2014 15 (Supplementary Table 1). Virus stocks were prepared in feline embryo (FEA) cells 17. Field isolates were used at passage 4 or less. Vaccine virus (FCV-F9; provided by MSD Animal Health) was used at passage 2.

Phylogenetic analysis

Methods for nucleic acid isolation, reverse transcription, PCR amplification, purification and sequencing were as previously described 18, leading to a final PCR product of 529 nucleotides of the immunodominant region of the FCV capsid gene 19, corresponding to residues 6406-6934 of the FCV-F9 genome (Genbank M86379). Resulting sequences were aligned and pairwise p-distances and Neighbour-Joining trees calculated (MEGA6) 20-22. All ambiguous positions associated with the quasi-species nature of FCV were discounted for each sequence pair.

Virus neutralisation (VN)

Sixty field viruses were used in VNs. Forty isolates from 2001 were used; 7 (17.5%) were from cats showing acute disease (URTD and/or mouth ulcers), 30 (75%) from healthy cats and 3 (7.5%) had no clinical data (Supplementary Table 1). Of 48 isolates collected in 2013/14, a subset of 20 was used in this study. To maintain the same approximate clinical ratio as for the 2001 isolates, they were similarly stratified and 4 (20%) isolates with acute disease and 16 (80%) from healthy cats were randomly selected for inclusion. Viruses from clinically normal cats were included as these are still likely to be virulent 23. Plasma (hereafter anti-serum) was collected and pooled from four specific pathogen free cats vaccinated sub-cutaneously with 10 commercial doses of Nobivac ®Tricat Trio (FCV-F9) at 8-9 weeks of age, and again 4 weeks later, as part of a vaccine safety study conducted by the funder15. Blood samples were taken 3 weeks after the second vaccination. Virus neutralisation tests were performed using a constant virus, varying anti-serum method as previously described15, 24. An estimated concentration of 100 TCID50  of virus was used in each assay and viral back titration was used to ensure the viral titre fell into the accepted range of 32-320 TCID50 25. Antibody titres were expressed as 50% end points26. An internal FCV-F9 homologous control was included in each experiment; individual tests were only considered valid if the neutralising titre for this control was within two-fold of the mean homologous control titre across all experiments 27. Antibody units (AUs) were also calculated with 1 AU being the highest dilution of FCV-F9 anti-serum that neutralised 32–320 TCID50 of homologous virus in 50% of cultures, based on the average FCV-F9 titre for all experiments27, 28.

Statistical analysis

Levine’s homogeneity of variance and Mann-Whitney tests were used to compare the variance and distribution of neutralisation titres across the two populations of isolates (2001 versus 2013/14) and between clinical and non-clinical isolates. Fisher’s exact test was used to compare the proportion of samples neutralised by different levels of AUs. Intra- and/or inter-population genetic distances were compared using Mann-Whitney test.

Results

Sequencing and phylogenetic analysis

Partial capsids could not be amplified from five of the isolates used in viral neutralisation, presumably due to a mismatch of the primers used 18, 29. Sequences for the remaining 55 isolates are available in GenBank - Accession Numbers KX257491–617 (previously published 15) and MH674290-347 (see Supplementary Table 2). Phylogenetic analysis of the 55 sequences (together with FCV-F9) showed a typically radial distribution dominated by 52 distinct strains (pairwise genetic distance >20%, bootstrap values <80%; 30) (Figure 1), with strains from both studies intermingled. Similarly, the sequence for FCV-F9 appeared integral to this phylogeny. This overall lack of apparent temporal clustering was supported by there being no significant differences in the range of genetic distances produced either within each studied population (intra-population distances for 2001 and 2013/14 isolates), or between them (inter-population distances for comparing 2001 and 2013/14 isolates) or between each population and FCV-F9 (P>0.05 for all comparisons) (Supplementary Figure 1). Only two clades (a and b) were evident, containing possible variants of individual strains (pairwise genetic distance <20%, bootstrap values >80%); clade b contained two isolates from Oxfordshire (isolated 13 years apart) suggesting possible local circulation of this strain over some years.

Virus neutralisation

Neutralisation profiles were obtained for 59 of the 60 isolates (Supplementary Table 1, Figure 2); one isolate repeatedly failed internal experimental controls. All 59 isolates were neutralised by anti-serum at a 1 in 4 dilution. We advise caution to be applied to the interpretation of results obtained at this dilution since, although our previous experiments suggest otherwise, low-levels of non-specific neutralisation may be observed. A 1 in 8 dilution neutralised 97 and 100% of the 2001 and 2013/14 isolates respectively and a 1 in 16 dilution neutralised 87 and 75% of isolates respectively. Importantly, there was no significant difference in variance between the titres for the two populations (2001 versus 2013/14, P=0.97) and no significant difference in the distribution of titres either across these two temporally separated populations (P=0.46), or between isolates from clinically affected and clinically normal cats (P=0.36) (Figure 2). The percentage of isolates neutralised by 5, 10 and 20 AUs were 36, 59 and 64% respectively for the 2001 isolates, and 25, 45 and 55% respectively for the 2013/14 isolates (Figure 2). These differences were not statistically significant (P=0.16).

Discussion

Some authors have suggested that vaccines containing older FCV strains may be less relevant for the control of current field viruses 12-14. Here, we test this hypothesis by directly comparing the ability of FCV-F9 anti-serum to neutralise two representative UK populations of FCV isolated 12-13 years apart. Our results showed FCV-F9 antiserum induced broad cross-neutralisation *in vitro*, showing no significant difference in the neutralisation of either population. These observations would suggest that FCV-F9 antiserum is not becoming less cross-reactive, and are consistent with a challenge experiment in which cats vaccinated with FCV-F9 were broadly protected when challenged with a recent field isolate 31, 32. The differences between our results and those of others are likely attributable to variations in methodology including the use of non-representative isolates 12. In addition, the majority of earlier studies have not directly compare temporally separated, spatially representative isolates using the same antisera.

How can this apparent antigenic stability be reconciled with both rapid evolution of FCV in acute infection 6, and the high levels of FCV genetic variability observed particularly in colonies of cats 29, 33? Consistent with previous studies, our phylogenetic analysis highlighted a radial phylogeny containing many strains 3, 4, 29, 34 . However, we observed no phylogenetic clustering, with sequences from both time points intermingled in the phylogeny, to which FCV-F9 remained integral 4, 29, 34, 35. Similarly, distance comparisons between both sets of isolates and FCV-F9 showed neither population of field isolates was significantly more variable to FCV-F9 than to itself. These observations suggest that the capsid of FCV is not evolving in a linear (molecular clock-like) fashion, as is typical for some other rapidly evolving viruses like influenza 15, 36. These genetic observations correlate with our neutralisation data, being linked through our use of sequences from the immunodominant region of the FCV capsid, where most known neutralisation epitopes reside 19, 37. Our working hypothesis is that whilst this region can evolve quickly in response to immune selection in individual animals 6, 38, structural constraints on the capsid may prevent its continued evolution and broader antigenic escape at the population level 23. To further understand these observations it will be necessary to expand these analyses to include full FCV genome sequences, including older isolates collected closer in time to when FCV-F9 was isolated around 1958.

Using 20AUs as a cut-off, we observed neutralisation of 64% and 55% of 2001 and 2013/14 isolates respectively. Older UK studies reported 54% 39 and 74% 27 of field isolates neutralised by FCV-F9 antiserum at the 20AU cut-off. Our previous study using the same anti-serum used here, but with a larger number of isolates, showed 50% were neutralised by 20AU 15. However, our 2001 study using different anti-serum showed only 25% of isolates to be neutralised by 20AU 16, highlighting how results can vary even when AUs are used to correct for differences in anti-serum titres. Although AUs are undoubtedly useful for comparing results between studies, the method used here where isolates are directly compared within a single study should perhaps be preferable.

Conclusions

Our studies demonstrate that although FCV field strains are very variable, they do not appear to be evolving away from the FCV-F9 vaccine strain either antigenically or genetically. Although our *in vitro* VN study cannot equate with clinical protection, together with the phylogenetic data, it may suggest a mechanism for why FCV-F9 antisera still remain broadly cross-reactive *in vitro* against FCV field isolates.

Conflict of interest

This project was funded by MSD-Animal Health who markets a live attenuated FCV vaccine containing the FCV-F9 virus.

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Ethics Statement

Ethical approval for both the 2001 and 2013/2014 studies was obtained from the Veterinary Research Ethics Committee, University of Liverpool. Informed consent was obtained from all participating owners involved in both studies.

References

1. Radford AD, Addie D, Belak S, et al. Feline calicivirus infection. ABCD guidelines on prevention and management. *J Feline Med Surg*. 2009; 11: 556-64.

2. Geissler K, Schneider K, Platzer G, Truyen B, Kaaden OR and Truyen U. Genetic and antigenic heterogeneity among feline calicivirus isolates from distinct disease manifestations. *Virus Res*. 1997; 48: 193-206.

3. Glenn M, Radford AD, Turner PC, et al. Nucleotide sequence of UK and Australian isolates of feline calicivirus (FCV) and phylogenetic analysis of FCVs. *Vet Microbiol*. 1999; 67: 175-93.

4. Henzel A, Sa e Silva M, Luo S, Lovato LT and Weiblen R. Genetic and phylogenetic analyses of capsid protein gene in feline calicivirus isolates from Rio Grande do Sul in southern Brazil. *Virus Res*. 2012; 163: 667-71.

5. Kreutz LC, Johnson RP and Seal BS. Phenotypic and genotypic variation of feline calicivirus during persistent infection of cats. *Vet Microbiol*. 1998; 59: 229-36.

6. Radford AD, Turner PC, Bennett M, et al. Quasispecies evolution of a hypervariable region of the feline calicivirus capsid gene in cell culture and in persistently infected cats. *J Gen Virol*. 1998; 79 ( Pt 1): 1-10.

7. Seal BS. Analysis of capsid protein gene variation among divergent isolates of feline calicivirus. *Virus Res*. 1994; 33: 39-53.

8. Kalunda M, Lee KM, Holmes DF and Gillespie JH. Serologic classification of feline caliciviruses by plaque-reduction neutralization and immunodiffusion. *Am J Vet Res*. 1975; 36: 353-6.

9. Kahn DE and Hoover EA. Feline caliciviral disease: experimental immunoprophylaxis. *Am J Vet Res*. 1976; 37: 279-83.

10. Pedersen NC and Hawkins KF. Mechanisms for Persistence of Acute and Chronic Feline Calicivirus Infections in the Face of Vaccination. *Veterinary Microbiology*. 1995; 47: 141-56.

11. Bittle JL, York CJ, Newberne JW and Martin M. Serologic Relationship of New Feline Cytopathogenic Viruses. *American Journal of Veterinary Research*. 1960; 21: 547-50.

12. Addie D, Poulet H, Golder MC, et al. Ability of antibodies to two new caliciviral vaccine strains to neutralise feline calicivirus isolates from the UK. *Vet Rec*. 2008; 163: 355-7.

13. Lauritzen A, Jarrett O and Sabara M. Serological analysis of feline calicivirus isolates from the United States and United Kingdom. *Vet Microbiol*. 1997; 56: 55-63.

14. Wensman JJ, Samman A, Lindhe A, Thibault JC, Berndtsson LT and Hosie MJ. Ability of vaccine strain induced antibodies to neutralize field isolates of caliciviruses from Swedish cats. *Acta Vet Scand*. 2015; 57: 86.

15. Afonso MM, Pinchbeck GL, Smith SL, et al. A multi-national European cross-sectional study of feline calicivirus epidemiology, diversity and vaccine cross-reactivity. *Vaccine*. 2017; 35: 2753-60.

16. Porter CJ, Radford AD, Gaskell RM, et al. Comparison of the ability of feline calicivirus (FCV) vaccines to neutralise a panel of current UK FCV isolates. *J Feline Med Surg*. 2008; 10: 32-40.

17. Jarrett O, Laird HM, Crighton GW, Jarrett WF and Hay D. Advances in feline leukemia. *Bibl Haematol*. 1968; 30: 244-54.

18. Hou J, Sanchez-Vizcaino F, McGahie D, et al. European molecular epidemiology and strain diversity of feline calicivirus. *Vet Rec*. 2016; 178: 114-5.

19. Radford AD, Willoughby K, Dawson S, McCracken C and Gaskell RM. The capsid gene of feline calicivirus contains linear B-cell epitopes in both variable and conserved regions. *J Virol*. 1999; 73: 8496-502.

20. Nei M and Kumar S. *Molecular evolution and phylogenetics*. Oxford ; New York: Oxford University Press, 2000, p.xiv, 333 p.

21. Tamura K, Nei M and Kumar S. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc Natl Acad Sci U S A*. 2004; 101: 11030-5.

22. Tamura K, Stecher G, Peterson D, Filipski A and Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol*. 2013; 30: 2725-9.

23. Povey RC and Hale CJ. Experimental infections with feline caliciviruses (picornaviruses) in specific-pathogen-free kittens. *J Comp Pathol*. 1974; 84: 245-56.

24. Dawson S, McArdle F, Bennett D, et al. Investigation of vaccine reactions and breakdowns after feline calicivirus vaccination. *Vet Rec*. 1993; 132: 346-50.

25. Povey RC and Johnson RH. A standardized serum neutralization test for feline viral rhinotracheitis. II. The virus-serum system. *J Comp Pathol*. 1969; 79: 387-92.

26. Reed LJ and Muench H. A simple method of estimating fifty per cent endpoints. *American Journal of Epidemiology*. 1938; 27: 493–7.

27. Dawson S, McArdle F, Bennett M, et al. Typing of feline calicivirus isolates from different clinical groups by virus neutralisation tests. *Vet Rec*. 1993; 133: 13-7.

28. Kapikian AZ, Conant RM, Hamparian VV, et al. Rhinoviruses: A Numbering System. *Nature*. 1967; 213: 761.

29. Coyne KP, Christley RM, Pybus OG, Dawson S, Gaskell RM and Radford AD. Large-scale spatial and temporal genetic diversity of feline calicivirus. *J Virol*. 2012; 86: 11356-67.

30. Radford AD, Bennett M, McArdle F, et al. The use of sequence analysis of a feline calicivirus (FCV) hypervariable region in the epidemiological investigation of FCV related disease and vaccine failures. *Vaccine*. 1997; 15: 1451-8.

31. Almeras T, Schreiber P, Fournel S, et al. Comparative efficacy of the Leucofeligen FeLV/RCP and Purevax RCP FeLV vaccines against infection with circulating feline Calicivirus. *BMC Vet Res*. 2017; 13: 300.

32. Lesbros C, Martin V, Najbar W, et al. Protective Efficacy of the Calicivirus Valency of the Leucofeligen Vaccine against a Virulent Heterologous Challenge in Kittens. *Vet Med Int*. 2013; 2013: 232397.

33. Radford AD, Dawson S, Ryvar R, et al. High genetic diversity of the immunodominant region of the feline calicivirus capsid gene in endemically infected cat colonies. *Virus Genes*. 2003; 27: 145-55.

34. Sato Y, Ohe K, Murakami M, et al. Phylogenetic analysis of field isolates of feline calcivirus (FCV) in Japan by sequencing part of its capsid gene. *Vet Res Commun*. 2002; 26: 205-19.

35. Prikhodko VG, Sandoval-Jaime C, Abente EJ, et al. Genetic characterization of feline calicivirus strains associated with varying disease manifestations during an outbreak season in Missouri (1995-1996). *Virus Genes*. 2014; 48: 96-110.

36. Gojobori T, Moriyama EN and Kimura M. Molecular clock of viral evolution, and the neutral theory. *Proc Natl Acad Sci U S A*. 1990; 87: 10015-8.

37. Tohya Y, Yokoyama N, Maeda K, Kawaguchi Y and Mikami T. Mapping of antigenic sites involved in neutralization on the capsid protein of feline calicivirus. *J Gen Virol*. 1997; 78 ( Pt 2): 303-5.

38. Coyne KP, Gaskell RM, Dawson S, Porter CJ and Radford AD. Evolutionary mechanisms of persistence and diversification of a calicivirus within endemically infected natural host populations. *J Virol*. 2007; 81: 1961-71.

39. Knowles JO, Dawson S, Gaskell RM, Gaskell CJ and Harvey CE. Neutralisation patterns among recent British and North American feline calicivirus isolates from different clinical origins. *Vet Rec*. 1990; 127: 125-7.

Figure Legends

Figure 1

Unrooted Neighbour-Joining tree of the 56 partial FCV capsid sequences used in this study (including FCV-F9; GenBank Accession Number M86379) . The evolutionary distances were computed using the p-distance method 20 and are in the units of the number of base differences per site (see 0.05 scale bar which equates to 5 changes per 100 bases). All codon positions were included. Therewere 432 nucleotide positions in the final dataset. The isolates are numbered as in Supplementary Table 1, which includes a two letter code for the geographical area, two digits for the isolate number and two digits for the year of collection; isolates from each of the two studies are also differentiated by colour (see key). \*Represents isolates from cats with acute disease (URTD/+ ulcers).. Clades represented by more than a single sequence (<20% capsid divergence, ≥ 80% bootstrap values) are boxed, additionally labelled a and -b, and the intra-clade diversity indicated. Bootstrap values of less than 80% are not shown.

Figure 2

Results of virus neutralisation. The percentage of isolates neutralised by each dilution of FCV-F9 anti-sera in the 2-fold dilution series (or by the midpoint between 2-fold dilutions as calculated by the Reed-Muench equation 26) are shown. The results for 5 isolates fell outside these groupings (see Supplementary Table 1\*\*); in which case the result was rounded down to the nearest grouping. Calculated levels of 5, 10 and 20 AU (titres of 1 in 200, 1 in 100 and 1 in 50, respectively) are indicated.

Supplementary Material

Supplementary Figure 1

Evolutionary divergence between sequences. Pairwise nucleotide distances between sequences were calculated using the Maximum Composite Likelihood model in MEGA621. The analysis involved 56 nucleotide sequences (including FCV-F9), each with 432 nucleotides in the final data set.

Distances are shown between isolates within each time period (intra-group distances), between isolates in each group (inter-group distances) and between isolates in each group and F9.

Supplementary Table 1

Isolate details and results of virus neutralisation using FCV-F9 anti-sera. The isolate name includes a two letter code for the geographical area, two digits for the isolate number and two digits for the year of collectionVirus neutralisation is expressed as the 50% end-point as calculated using the Reed-Muench equation 26 . Clinical isolates are indicated by \* and also highlighted in lilac. The results for 5 isolates \*\* fell outside either a 2-fold dilution or the mid-point between two dilutions. Where this occurred, the result was rounded down to the nearest real value (plotted titres) as calculated by the Reed-Muench equation and this was plotted in Figure 2. One virus repeatedly failed the VN assay (marked as ‘fail’). The titre of the virus in the neutralisation test is also shown (the allowable range being 32-320 TCID50. n/a indicates this data was not collected.

Supplementary Table 2

Isolate details. Isolate names and codes used in this and previous papers 15, 16 are indicated to allow the reader to compare results between papers. GenBank accession numbers for the capsid sequence from isolates used in the phylogenetic analysis are detailed. DNP = Did not PCR.