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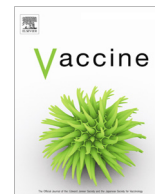
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## A multi-national European cross-sectional study of feline calicivirus epidemiology, diversity and vaccine cross-reactivity

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### ABSTRACT

**Background:** Feline calicivirus (FCV) is an important pathogen of cats for which vaccination is regularly practised. Long-term use of established vaccine antigens raises the theoretical possibility that field viruses could become resistant. This study aimed to assess the current ability of the FCV-F9 vaccine strain to neutralise a randomly collected contemporary panel of FCV field strains collected prospectively in six European countries.

**Methods:** Veterinary practices (64) were randomly selected from six countries (UK, Sweden, Netherlands, Germany, France and Italy). Oropharyngeal swabs were requested from 30 (UK) and 40 (other countries) cats attending each practice. Presence of FCV was determined by virus isolation, and risk factors for FCV shedding assessed by multivariable logistic regression. Phylogenetic analyses were used to describe the FCV population structure. *In vitro* virus neutralisation assays were performed to evaluate FCV-F9 cross-reactivity using plasma from four vaccinated cats.

**Results:** The overall prevalence of FCV was 9.2%. Risk factors positively associated with FCV shedding included multi-cat households, chronic gingivostomatitis, younger age, not being neutered, as well as residing in certain countries. Phylogenetic analysis showed extensive variability and no countrywide clusters. Despite being first isolated in the 1950s, FCV-F9 clustered with contemporary field isolates. Plasma raised to FCV-F9 neutralized 97% of tested isolates (titres 1:4 to 1:5792), with 26.5%, 35.7% and 50% of isolates being neutralized by 5, 10 and 20 antibody units respectively.

**Conclusions:** This study represents the largest prospective analysis of FCV diversity and antigenic cross-reactivity at a European level. The scale and random nature of sampling used gives confidence that the FCV isolates used are broadly representative of FCVs that cats are exposed to in these countries. The *in vitro* neutralisation results suggest that antibodies raised to FCV-F9 remain broadly cross-reactive to contemporary FCV isolates across the European countries sampled.

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### 1. Introduction

Feline calicivirus (FCV) is a common pathogen of cats causing oral and upper respiratory tract disease (URTD) [1]. It has a single-stranded, positive-sense RNA genome [2], the plasticity of which is important for antigenic evolution, viral persistence [3,4] recombination [5,6], and the sporadic outbreaks of highly virulent FCV strains causing severe disease [1,7,8]. Despite high levels of variability, FCV strains are generally considered to comprise one diverse genogroup with a radial phylogeny and little evidence for sub-species clustering [9–14].

This diverse genogroup is mirrored by a single diverse serotype; although individual strains are distinguishable antigenically, they generally show some cross-reactivity [15–18], allowing the development of several FCV vaccines based on different antigens [1]. Whilst vaccines reduce clinical signs, none are licensed to reduce virus shedding post-challenge and FCV infection remains highly prevalent in both vaccinated and unvaccinated populations [1]. Most live vaccines include FCV-F9 [19,20], whereas inactivated vaccines commonly include strains FCV-255, or a combination of FCV-431 and FCV-G1 [18,21,22]. These vaccine antigens are chosen based on their ability to induce broadly cross-reactive antisera against contemporary isolates circulating at the time of vaccine development [17,20,22]. The widespread use of such vaccines together with the high adaptability of FCV raises the theoretical

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possibility that vaccine resistant strains may evolve over time. Whilst some studies have supported this hypothesis [23–25], others have not [26].

Here we describe the antigenic and genetic relationships between FCV-F9 and a representative panel of currently circulating FCV strains, obtained from randomly selected veterinary practices across six European countries.

## 2. Materials and methods

### 2.1. Ethical statement

Ethical approval was from the Veterinary Research Ethics Committee, University of Liverpool. Informed consent was obtained from participating owners.

### 2.2. Recruitment

Samples were collected between October 2013 and May 2014 from cats attending veterinary practices in the UK, France, Italy, Netherlands, Sweden and Germany.

In the UK, three Unitary Authorities (UAs) were randomly chosen from each of the nine regions of England, as well as from Wales, Scotland and Northern Ireland. Geographically remote islands were also selected (Western Isles, Orkney, Shetland, Anglesey, Isle of Wight, Scilly Isles, Isle of Man, Channel Islands) based on convenience. From each of these 44 regions, a small animal practice was randomly selected from the Royal College of Veterinary Surgeons database. The remaining five countries were chosen based on convenience, divided into five regions based on official divisions and/or local geography, and a single practice randomly selected from each. If chosen practices declined to participate, a further practice was randomly selected. This process was repeated up to three times until a practice in each region agreed to take part.

### 2.3. Field isolates

There is much debate regarding the most appropriate FCV isolates to use for assessment of *in vitro* neutralisation. Several studies have used isolates obtained by convenience from diagnostic laboratories to represent pathogenic viruses [23–25]; lack of random sampling means such results may not be generalizable to the wider population [24]. Here we sample sick and healthy cats randomly to ensure our results are representative of the sampled population. The occasional description of non-pathogenic FCV strains [27] requires us to justify the inclusion of isolates from healthy animals. In this regard, it should be noted that FCV isolates from healthy cats can still be pathogenic: virulent FCV continues to be shed from cats recovered from acute disease [27], and seropositive cats previously exposed to vaccine or field virus may shed virus in absence of clinical signs when subsequently challenged with virulent virus [19]. Indeed, experimental challenge has confirmed that FCV from healthy cats can recreate typical disease [27].

In each practice, veterinary surgeons were asked to collect oropharyngeal swabs from the next 30 (UK) or 40 (other countries) cats presented at their surgery regardless of reason for presentation (diseased or healthy). Random recruitment of practices and random sampling of cats based on attendance at these practices were used to ensure results could be generalised to the sampled population, and is in contrast to an earlier study by the authors where sampling was by convenience [10].

Swabs were collected into virus transport medium, stored at  $-20^{\circ}\text{C}$  before shipping to the laboratory. The veterinary surgeon and owner were asked to complete a short questionnaire capturing demographic data, vaccination history and information about cur-

rent respiratory disease, mouth ulcers and chronic gingivostomatitis (CGS).

### 2.4. Viral isolation (VI)

Feline calicivirus was isolated using standard techniques [28] based on presence of typical cytopathic effect (CPE). Samples were only considered negative after two passages [29].

### 2.5. RNA extraction and reverse transcription-PCR

Viral RNA was extracted from positive cell cultures (second passage or less) (Viral RNA mini-kit; Qiagen). One negative control (mock infected cells) was included for each three samples. Reverse transcription was performed using 200 ng random hexamers (Superscript III, Life Technologies). A 529-nucleotide region of the capsid gene, equivalent to residues 6406–6934 of FCV-F9 (GenBank M86379) and incorporating immunodominant regions C and E [3,30], was amplified according to manufacturer's guidelines (Reddy-Mix; Thermo scientific) and published protocols using 25 pmoles of each primer per 50  $\mu\text{l}$  reaction [10]. In addition, 486-nucleotides from the 3' end of the FCV polymerase gene were also sequenced as previously described [10].

### 2.6. Nucleotide sequencing and phylogenetic analysis

Amplicons were purified (QIAquick; Qiagen), quantified (Nanodrop; Genequant) and sequenced (Source Bioscience; Nottingham). Forward and reverse sequences were aligned (ChromasPro; Technelysium), and pairwise p-distances and neighbour-joining trees (1000 bootstrap replicates) calculated using MEGA7. A threshold of 20% uncorrected nucleotide distance was used to define distinct strains [31,32].

### 2.7. Epidemiological analysis

Prevalence estimates with 95% confidence intervals were determined (EpiTools; AusVet) based on results of VI. Data from questionnaires were used to examine risk factors and associations with FCV carriage. Univariable and multivariable multilevel logistic regression allowing for clustering within practice was conducted using MLwiN (v2.1, University of Bristol). Potential risk factors included country, cat's age, gender, breed, lifestyle, vaccination status, vaccine strain, neutering status, presence of mouth ulcers, URD signs, CGS and number of cats in the household. Variables with  $P$ -values  $<0.25$  in initial univariable analysis were considered in the multivariable model retaining variables with Wald  $P$ -values  $<0.05$ .

### 2.8. Isolates and plasma for viral neutralisation (VN) testing

Isolates for VN testing were randomly selected with stratification, approximately half were from the UK, the remainder from other participating countries. There is no approved standard for producing immune reagents for FCV neutralisation studies. Conventional FCV vaccination induces insufficient neutralisation titres [33], such that previous studies have used infection with vaccine viruses to produce test sera [23–26]. This will likely impact on both the quantity and range of any measured immune response compared to vaccination, especially when the tested vaccines often contain inactivated antigens. The plasma used in this study was collected from animals used in a standard vaccine safety study conducted by the funders. Four specific pathogen free cats were vaccinated subcutaneously with 10 commercial doses of Nobivac® TricatTrio (FCV-F9 live-attenuated vaccine) at 8–9 weeks of age, and again four weeks later. Blood samples were taken three weeks

after the second vaccination. Whilst such a challenge regime will induce a quantitatively higher response than routine vaccination, the antigenic targets for the response should be broadly similar to those of routine vaccination. The plasma from all four cats was used as a pool for all tested isolates, and also separately for 10 randomly selected isolates.

### 2.9. Viral neutralisation assays

Virus neutralisation tests were performed using a constant virus, varying plasma method. Briefly, duplicate, serial twofold dilutions of plasma were incubated with 32–320 TCID<sub>50</sub> [34] of virus at 37 °C for 1 h before addition to FEA cells which had been plated 24 hours previously at approximately  $1 \times 10^4$  cells/well of a 96 well plate. Plates were observed for CPE at 48 h and 120 h. Antibody titres were expressed as 50% end points [35]. An internal FCV-F9 homologous control was included in each experiment. As

homologous antibody titres can vary between experiments, between serum from different cats and depending on the method of challenge, antibody units (AU) for each isolate were calculated using the titre of this internal control. One antibody unit (AU) is the highest plasma dilution neutralizing 100TCID<sub>50</sub> of homologous virus in 50% of cultures [15]. AUs were also calculated using the mean FCV-F9 titre of all experiments [26], excluding those in which the internal homologous FCV-F9 titre was >2-fold either side of the mean FCV-F9 titre for all experiments [33].

## 3. Results

### 3.1. Study sample

Fifty (27 UK, 23 mainland Europe) of the 64 recruited practices (78.1%) returned samples (Fig. 1). Of the 2140 samples requested, 1521 (71%) were received.



**Fig. 1.** Map showing the 64 recruited veterinary practices. Circles in red are the 50 practices that supplied samples during the study. Blue circles are the 14 recruited practices that did not send back samples during the study. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Table 1**  
Summary of samples, isolates and strains identified in each country.

Country	UK	France	Netherlands	Germany	Sweden	Italy
Samples (n)	686	187	99	175	205	149
FCV Positive n (%; CI)	58 (8.4%, 0.06–0.1)	11 (5.8%, 0.03–0.09)	16 (16.2%, 0.09–0.2)	25 (14.2%, 0.09–0.19)	17 (8.3%, 0.05–0.12)	8 (5.4%, 0.02–0.09)
FCV Capsid Sequence	56	11	16	19	17	10
Strains (n)	48	11	14	14	14	10

**Table 2**  
Multilevel multivariable logistic regression analysis (allowing for clustering within practice) of factors associated with FCV isolation in 1502 vet visiting cats from UK and mainland Europe. P-values below 0.05 considered significant.

Variable	Odds ratio	95% CI lower	95% CI upper	P-value
<i>Country</i>				
France	0.29	0.11	0.75	0.01
The Netherlands	Ref.			
Italy	0.234	0.087	0.631	0.004
Germany	0.72	0.31	1.65	0.44
Sweden	0.599	0.253	1.419	0.24
UK	0.434	0.213	0.88	0.02
<i>Neutered status</i>				
Yes	Ref.			
No	1.69	1.053	2.736	0.03
<i>Chronic gingivo-stomatitis</i>				
Yes	Ref.			
No	0.12	0.06	0.23	<0.001
<i>No. cats/household</i>				
1	Ref.			
2–3	1.75	1.10	2.79	0.02
4–10	2.82	1.49	5.31	0.001
>11	0.74	0.08	6.97	0.79
Age (month)	0.99	0.99	0.99	0.01
Level 2 variance (standard error)				0.035 (0.106)

### 3.2. Viral isolation

A total of 140 of 1521 samples tested positive for FCV (9.2%, 95% confidence interval (CI) 7.8, 10.8), ranging from 5.4% in Italy to 16.2% in the Netherlands (Table 1).

### 3.3. Epidemiological analysis

Questionnaires were not received for 1.2% of samples and therefore analysis was performed using 1502 questionnaire-sample matches. Nine of twelve predictor variables were significantly associated with FCV isolation in univariable analysis (data not presented). Of these, five remained significant on multivariable analysis (Table 2): Cats sampled in France, Italy and the UK were at a lower risk of shedding FCV than those from the Netherlands. Entire (non-neutered) cats were 1.7 times more likely to shed FCV than neutered cats, regardless of gender. Cats in multi-cat households were 1.7 (2–3 cats) and 2.8 (4–10 cats) times more likely to shed FCV than cats living alone. Cats with CGS were 8.3 times more likely to shed FCV than those without. Finally, each additional year of a cat's age reduced FCV shedding likelihood by 12%. Vaccination was not significantly associated with risk of FCV infection in the final model.

### 3.4. Sequencing and phylogenetic analysis

A total of 128 partial capsid consensus sequences were obtained from the 140 FCV isolates (Fig. 2; GenBank accession numbers KX257491–KX257617). The unamplified isolates typical of such experiments are presumed to be caused by primer mismatches [10,11]. In total, 110 strains (pairwise genetic distance >20%) were observed, ranging from 10 (Italy) to 48 (UK) (Table 1). Of these

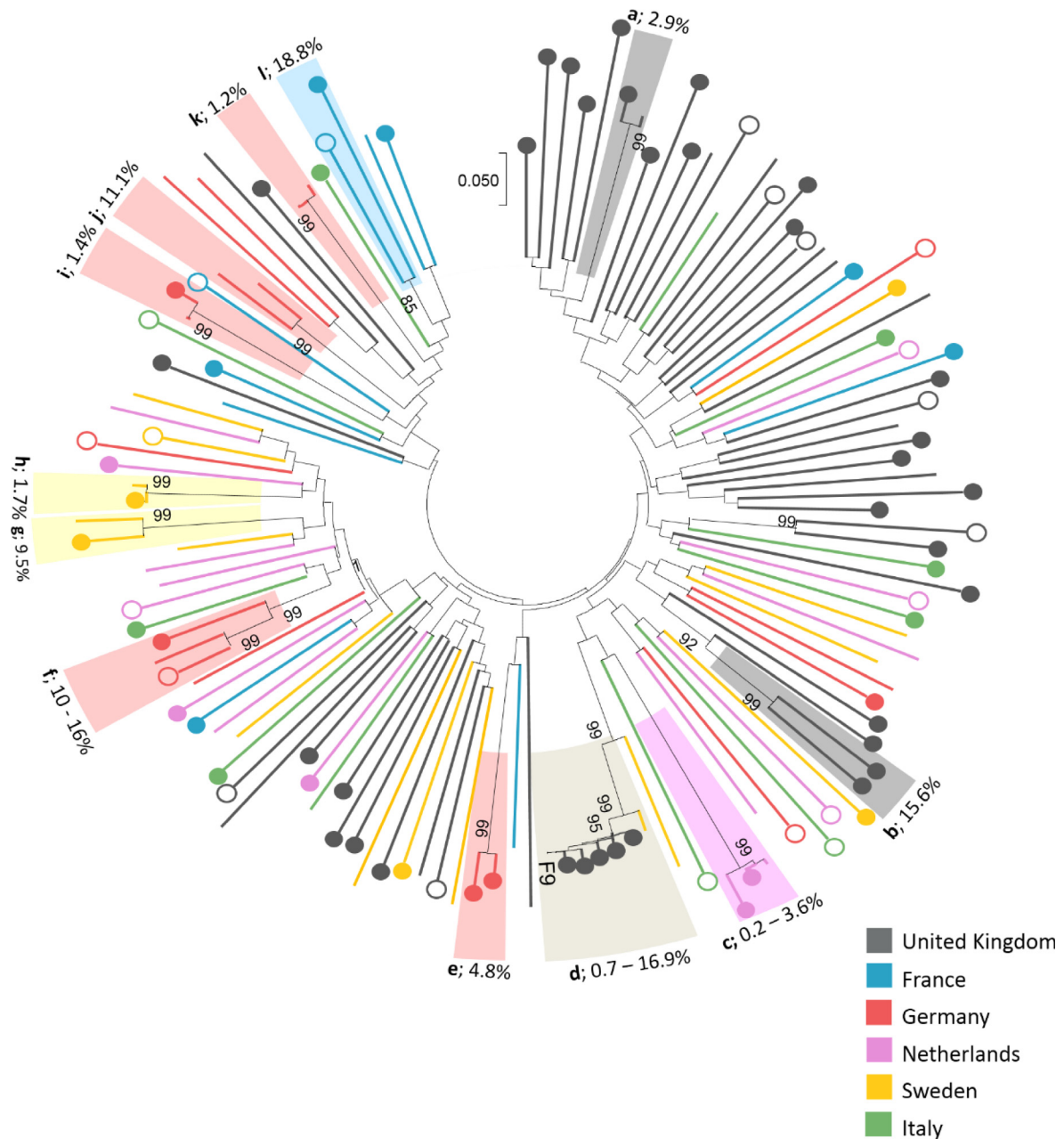
strains, only 10 were represented by more than one isolate (bootstrap values >80%; A to L on Fig. 2). The largest cluster included FCV-F9-like isolates from the UK and Sweden (D in Fig. 2). All other strains with more than one variant were restricted to individual practices, with no evidence for widespread or international transmission. Similar phylogenetic results were obtained for the polymerase gene (Supplemental File 1).

### 3.5. Viral neutralisation assays

The reproducibility of VN assays was assessed in two ways. Firstly, 10 field isolates were randomly repeated giving an average difference in neutralisation titres between repeats of 2.08, comparable to previous studies [34]. In addition, the mean homologous titre for the internal FCV-F9 control across 19 experiments was 1 in 1658 ± 345 standard error (data not presented).

Viral neutralisation was attempted in 121 of the 140 FCV isolates. In total, 98 VN tests were successfully completed (48 UK, 6 Sweden, 9 France, 16 Germany, 10 the Netherlands and 9 Italy; Supplemental File 2); the remaining 23 failed due to inability to regrow in cell culture, titration failure, or bacterial contamination.

Of these 98 FCV isolates, 95 (97%) were neutralized at titres ranging from 1:4 to 1:5792 (Fig. 3a; Supplemental File 2). Whilst group sizes precluded statistical analysis, the pattern of neutralisation appeared to be broadly similar when isolates from different clinical presentations were compared (Supplemental File 3). The VN results based on different countries are shown in Fig. 3b. When titres were standardised to homologous FCV-F9 titres derived within individual experiments, 26.5%, 35.7% and 50% of isolates were neutralized by 5, 10 and 20 AUs respectively. When using the same method as described previously [33], using only those experiments where the titre for the internal FCV-F9 control was within 2-fold of mean FCV-F9 titre across all experiments, 0%,



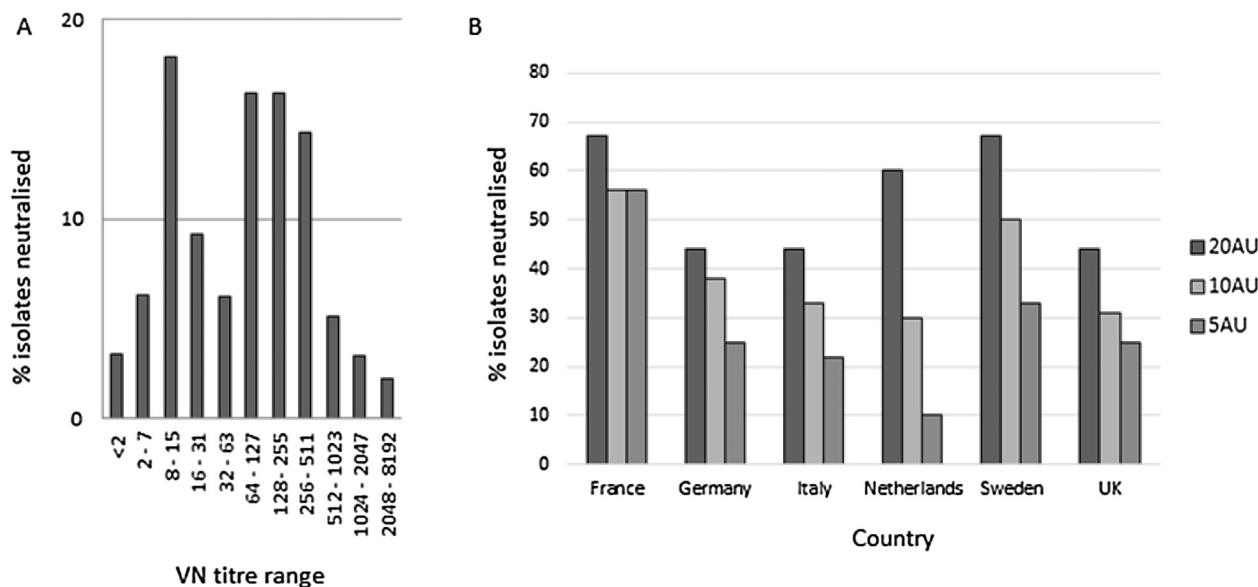
**Fig. 2.** Unrooted Neighbor-joining tree of 128 FCV partial capsid consensus sequences obtained from the national study (including the sequence of FCV vaccine strain F9 [GenBank accession No. M86379]). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown, only bootstrap values >80% are indicated. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree and relate to the distance bar. The evolutionary distances were computed using the Tamura-Nei method and represent the number of base substitutions per site. The rate variation among sites was modelled with a gamma distribution (shape parameter = 1). Codon positions included were 1st + 2nd + 3rd + Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 435 positions in the final dataset. The geographical origin of sequences is shown in different colours. Those strains represented by more than a single sequence (<20% capsid divergence) are boxed, additionally labelled A–L, and the intra-strain capsid diversity indicated next to the box; isolates in each box originate from the same country and veterinary practice with the exception of cluster D, where all sequences were collected in different practices and two different countries. Circles at the tip of the branch indicate isolates used in viral neutralisation testing: empty circles represent isolates from animals with either chronic or acute upper respiratory tract disease (URTD) and full circles represent isolates from animals either without URTD or no information in regards to clinical status. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

20% and 32% of 25 isolates were neutralized by 5, 10 and 20 AU respectively (Table 3).

In order to analyse the variability of plasma from the four cats, viral neutralizations with single cat plasma were undertaken for 10 random field isolates and FCV-F9 (Supplemental File 4). The plasma from each cat had demonstrable neutralizing ability to each isolate. However, there was variation in the order of individual cat responses, with some cats' plasma seemingly neutralising some viruses particularly well, and others less well.

#### 4. Discussion

Widespread use of individual vaccines is associated with a theoretical risk for the emergence of vaccine resistance strains, particularly for RNA viruses. Here we have undertaken the first multinational European study to assess the current *in vitro* cross reactivity of FCV-F9, first isolated over 40 years ago, and still one of the most frequently used vaccine antigens [17]. In order to maximise the generalizability of our findings to the European cat



**Fig. 3.** Results of viral neutralisation testing. A: VN titre ranges (% of isolates) for the current study. B: Percentage of isolates neutralized by 20, 10 and 5AU per country.

**Table 3**

Percentage of isolates neutralized by 20, 10 and 5AU for each different cut-off or analysis method used.

Cut-off	Number of VN results	20AU	10AU	5AU
AUs based on individual experiment FCV-F9 homologous control	98	50	36	27
AUs for those isolates where the titre for the internal F9 control was within 2-fold of mean F9 titre across all experiments	25	32	20	0

population, a cross-sectional survey sampling cats from randomly recruited veterinary practices was undertaken. This approach also provided an opportunity to assess the epidemiology and molecular epidemiology of FCV infection.

Consistent with previous studies, cats in multi-cat households, those with CGS, and younger cats, were more likely to shed FCV [26,36]. Chronic gingivostomatitis affects 0.7% of the population [37], with most affected cats testing FCV positive [29,36]. Previous studies have shown that FCV prevalence increases from around 10% in single-cat households to over 50% in some larger colonies [4,38]. These large colonies are believed to drive antigenic diversity as strain variants evolve under positive selection within a variable population immunity [4,39]. In addition, neutered cats were less likely to test positive for FCV regardless of age. This suggests behavioural changes associated with neutering, such as becoming less territorial, may lower FCV risk as has also been shown for feline immunodeficiency virus [40,41]. We also found that cats in some countries (the Netherlands) had a higher prevalence of FCV infection than those from others (France, Italy, UK). Whether this represents true population differences, or the relatively small sample sizes in some countries will need to be assessed further.

The phylogenetic analysis is broadly in agreement with previous national and international studies [9–11,14], highlighting a radial phylogeny with little evidence for sub-species clustering except viruses sharing immediate temporal or spatial links. As previously [4,11,42], FCV-F9 variants were found in this population, five from the UK and two from Sweden, of which four had been vaccinated with FCV-F9 attenuated vaccines <25 days prior to sampling, one was un-vaccinated for at least three years, and one a rescue cat that was presumed unvaccinated. The only time such vaccine-derived viruses are not observed is when recently vaccinated cats are excluded from the sampled population [10]. Our

findings are consistent with experimental studies showing occasional shedding of vaccine virus following live-FCV vaccination [20,43]. Looking at the diversity within this FCV-F9 clade, six of the seven strains were <3.6% distant from the FCV-F9 published sequence, suggesting they had not been replicating for long in the cat, consistent with recent vaccination history of most of these cats. In contrast, a Swedish isolate from a vaccinated cat (unknown strains), was 16.9% different to FCV-F9, possibly representing a rare persisting and evolving strain of FCV-F9 or an unrelated strain. Taken together, this confirms that whilst live vaccine viruses are occasionally shed following vaccination, they only seem to have a limited potential to persist in the general cat population.

The balance between antibody- and cell-mediated immunity in FCV protection is somewhat uncertain. Some cats exposed to previous FCV antigens show protection to heterologous challenge, even when there are no demonstrable *in vitro* antibodies to the new challenge [29], suggesting that other factors including cellular immunity contribute to protection. That said, it is still believed that there is sufficient correlation between antibody levels and protection, for *in vitro* virus neutralisation tests to remain the accepted method of assessing cross-reactivity [16,24–26]. Therefore, we have used a pool of plasma raised to 10 doses of FCV F9 vaccine, and demonstrated neutralising activity to the majority of this cross-sectional European panel of contemporary FCV isolates. These results are broadly similar to those observed in a similar cross-sectional study of FCV-F9 strain diversity in the UK in 2001 [26]. When results are expressed as antibody units to try and control for variations in sera production (infection vs vaccination overdose), and the between-cat variation, the percentage of isolates neutralized by 20, 10 and 5 AUs was similar to, or higher than, that from the earlier study in 2001 [26]. When taken together, this suggests antisera against FCV-F9 remains broadly cross-reactive

against recently circulating FCV strain diversity. This is consistent with our observation that, despite its age, FCV-F9 remains an integral part of this contemporary phylogeny, and suggesting that FCV may not evolve in a linear (“clock-like”) fashion, such as is typical for other rapidly evolving viruses [44,45].

These conclusions are in contrast with other studies suggesting the levels of FCV-F9 cross-reactivity have reduced over time [23–25]. However, two important methodological differences between studies make direct comparisons impossible. Firstly, previous studies used isolates collected by convenience from diagnostic laboratories; these should not be considered representative of those in the general population [24]. Secondly, previous studies have used infection rather than vaccination to produce antisera of sufficient titre for testing; differences in viral replication and antigen presentation between virus replicating locally in target tissues of the upper respiratory tract as opposed to the subcutaneous tissues at the site of vaccination, are likely to impact in albeit unknown ways, on the nature of the ensuing immune response, and this impact is likely to be greatest for viral antigens from inactivated vaccines. Here for the first time we used subcutaneous vaccination (albeit at 10× release dose), of a live vaccine, using a cross-sectional sample of contemporary FCV isolates to maximise the generalizability of our results. Clearly these *in vitro* results cannot be used to suggest the rate of cross-protection in the field. To facilitate better comparison between these studies in the future, we recommend the development of an internationally agreed study protocol as exists for some other viral vaccines.

### Conflict of interest

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

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### Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vaccine.2017.03.030>.

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