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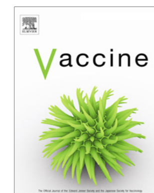

A multi-national European cross-sectional study of feline calicivirus epidemiology, diversity and vaccine cross-reactivity

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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 °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 current 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 25p-moles of each primer per 50 μl 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, URTD 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

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