Molecular characterization and virus neutralization patterns of severe, non-epizootic forms of feline calicivirus infections resembling virulent systemic disease in cats in Switzerland and in Liechtenstein

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Abstract

Feline calicivirus (FCV) infections are associated with oral ulceration, chronic stomatitis and a limping syndrome. Epizootic outbreaks of virulent systemic disease (VSD) have been reported in the USA and Europe. Here, the molecular characterization and neutralization patterns of FCV isolates from cases of severe, non-epizootic infection associated with skin ulceration and edema are presented. Samples from eleven symptomatic cats, four in-contact cats and 27 cats with no contact with symptomatic cats were collected and tested for FCV, feline herpesvirus-1 (FHV-1), feline leukemia virus (FeLV) and feline immunodeficiency virus (FIV). Phylogenetic analyses based on the capsid (VP1) gene of FCV and virus neutralization with antisera raised against four FCV vaccine strains were performed. Nine kittens and two adult cats in two shelters and two veterinary clinics in four geographically distinct locations in Switzerland and Liechtenstein were affected. The cats showed fever, tongue and skin ulceration, head and paw edema, and occasionally jaundice, generalized edema and dyspnea. All symptomatic cats tested FCV-positive but were negative for FHV-1, FeLV and FIV, with the exception of one FIV-positive kitten. All kittens of one litter and both adult cats died. The disease did not spread to cats in the environment. Cats in the environment displayed phylogenetically distinct, but related, FCV strains. Virus neutralization patterns suggested that some cases might have been potentially prevented by vaccination with the optimal vaccine strain. In conclusion, clinicians should be aware of severe, non-epizootic forms of FCV infections with initial clinical presentations similar to VSD.

Keywords: Feline calicivirus, virulent systemic disease, paw and mouth disease,

50 PCR, phylogenetic analysis, virus neutralization

51 ## Introduction

52 Feline calicivirus (FCV) is a highly infectious RNA virus of the family *Caliciviridae* and
53 one of the most common viral pathogens in cats worldwide (Radford et al., 2009).
54 The virus is detected in up to 40% of cats living in large groups (i.e. colonies or
55 shelters) and in about 10% of privately owned cats living alone or in small groups
56 (Bannasch and Foley, 2005; Coutts et al., 1994; Helps et al., 2005; Radford et al.,
57 2001; Wardley et al., 1974). FCV exhibits a remarkably high genetic evolution rate,
58 which is thought to result from genetic drift or recombination (Coyne et al., 2006a;
59 Coyne et al., 2007; Coyne et al., 2006c). Consequently, genetically diverse FCV
60 isolates can be isolated amongst naturally infected cats (Coyne et al., 2012). It has
61 been postulated that such genetic variation might favor the persistence of FCV in
62 groups of cats, leading to the emergence of novel strains (Coyne et al., 2006a;
63 Coyne et al., 2007; Coyne et al., 2006c).

64 Typical of vesivirus infections, FCV has been associated with vesicular disease
65 (Pesavento et al., 2008). Acute infections are characterized by transient fever and
66 ulcerations on the tongue and palate of affected cats (Radford et al., 2009). In more
67 severe cases, oral fauces, gingiva, lips and nasal philtrum may also be ulcerated.
68 Another clinical presentation of FCV infection is the limping syndrome associated
69 with transient lameness and acute synovitis (Radford et al., 2009). FCV has also
70 been assigned to the feline upper respiratory tract disease (URTD) complex;
71 however, classical signs of URTD in FCV-infected cats are often caused in
72 conjunction with other viral or bacterial pathogens (Binns et al., 2000; Cai et al.,
73 2002; Helps et al., 2005), and not all FCV isolates induce respiratory disease
74 following experimental challenge (Pesavento et al., 2008). FCV is also present in a

high proportion of cats displaying chronic lympho-plasmacytic gingivitis/stomatitis (Radford et al., 2009). This syndrome has so far not been successfully reproduced by experimental FCV infection (Knowles et al., 1991; Poulet et al., 2000) and is thought to represent an immune-mediated disease (Harley et al., 1999). In its most severe clinical form, FCV infection induces a highly contagious virulent systemic disease (VSD), which is characterized by a systemic inflammatory response syndrome (Pedersen et al., 2000). The disease involves internal organs as well as skin and mucous membranes. Affected cats show edema and skin ulceration, mainly around the head and limbs, and occasionally jaundice, dyspnea and bleeding tendencies (Coyne et al., 2006b; Pedersen et al., 2000; Radford et al., 2009; Schorr-Evans et al., 2003; Schulz et al., 2011). Epizootic outbreaks of VSD were first reported in cats in North America, but subsequently also in Europe (Coyne et al., 2006b; Hurley et al., 2004; Pedersen et al., 2000; Reynolds et al., 2009; Schorr-Evans et al., 2003; Schulz et al., 2011). The outbreaks usually occur in multi-cat environments and have been characterized by rapid onset and spread and high mortality (Radford et al., 2009). Published data suggest that these highly virulent strains emerge independently from genetically distinct FCV strains (Coyne et al., 2006b; Ossiboff and Parker, 2007; Reynolds et al., 2009; Schulz et al., 2011), but attempts to identify genetic patterns within the viral genome that define the highly virulent FCV biotype have been inconclusive (Abd-Eldaim et al., 2005; Foley et al., 2006; Prikhodko et al., 2014; Rong et al., 2006). Controversial results have been published concerning the protective effect of FCV vaccination against VSD. Most naturally infected cats developed VSD despite regular vaccination (Hurley et al., 2004; Schorr-Evans et al., 2003). However, experimental infection with a virulent-systemic FCV isolate resulted in a milder, self-limiting course in cats vaccinated with the FCV vaccine strain F9 when compared to unvaccinated cats (Pedersen et al., 2000).

In 1972, Cooper and Sabine described a cat with paw edema, oral lesions and skin ulcerations and called the syndrome 'paw and mouth disease' (Cooper and Sabine, 1972); FCV was isolated from tongue and paw lesions of the affected cat. The initial clinical presentation of this syndrome was similar to that reported as VSD, but the syndrome lacked high mortality, obvious organ involvement and epizootic disease spread. In the present case series, we report eleven cases of severe, non-epizootic forms of FCV infections associated with ulcerative lesions on the head and limbs and cutaneous edema that occurred in four unrelated geographic locations in Switzerland and Liechtenstein. The study describes clinical data from the cases and presents the molecular characterization and analysis of susceptibility to neutralization of the FCV isolates from the affected cats.

Material and Methods

Case definition, sample and data collection. Cases were included when they met the following criteria: 1) ulcerative lesions on the head and limbs and/or the presence of cutaneous edema; 2) the detection of FCV in oropharyngeal cytobrushes from the affected cats and, if available, in blood, skin lesions and organs; 3) the exclusion of FHV-1 infection; and 4) the isolation of a similar FCV isolate (> 99% nucleotide identity in a 1616 bp fragment of the ORF2) amongst the cats if several cats were affected. A total of eleven symptomatic cats from four different locations in Switzerland and Liechtenstein were included and signalment, vaccination status and clinical signs were recorded. Samples were collected as indicated in Table 1. In addition, an oropharyngeal cytobrush from the queen and three cats in contact with the affected litter in shelter 1 and from 27 cats with no direct contact to the affected litter in shelter 1 were obtained. In shelter 2, samples were collected from case 7 at the time of disease and from cases 7 and 8 two months later (Table 1). Only the

126 latter samples were available for virus neutralization assays and phylogenetic
127 analyses (see below).
128 ***Sample processing and nucleic acid extraction.*** Oropharyngeal cytobrushes and
129 swabs from cases 7 to 11 were collected into 300 µl of sterile viral transport medium.
130 The medium consisted of 200 ml bi-distilled sterile water, 4 ml HEPES-Buffer 1 M
131 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), 25 ml Dulbecco's MEM 10x
132 (Biochrom, Berlin, Germany), 25 ml heat inactivated fetal calf serum (FCS, Charge
133 DO2303P, Origin South America, Bio Concept, Allschwil, Switzerland), 3 ml 100 x
134 Antibiotic-Antimycotic (Gibco, Life Technologies, Lucerne, Switzerland) and 4 ml
135 sodium hydrogen bicarbonate 7.5% (Merck, Darmstadt, Germany) at a pH of 7 that
136 was adjusted using 1 M sodium hydroxide (Merck). Samples were stored at 4°C prior
137 to shipping to the laboratory by priority mail and were processed within 96 h after
138 collection. The samples from cases 1 to 6 were collected without viral transport
139 medium; 300 µl of sterile phosphate buffered saline (PBS) were added to these
140 samples upon arrival at the laboratory and the samples were processed within 12 h
141 after collection. All cytobrushes/swabs were incubated for 10 min at 40°C and then
142 turned upside down and centrifuged for 1 min at 6,440 x g. For cases 1 to 6, 100 µl of
143 the supernatant from the oropharyngeal cytobrush were used for virus isolation (see
144 below) and 200 µl were used for total nucleic acid (TNA) extraction. For cases 7 to
145 11, supernatants from the oropharyngeal cytobrush and the nasal and conjunctival
146 swabs were pooled and 400 µl used for virus isolation and 2 x 200 µl for TNA
147 extraction. TNA extraction was performed from 200 µl of the oropharyngeal
148 cytobrush, conjunctival and nasal swab supernatant, 200 µl of cell culture
149 supernatant and from 100 µl of EDTA blood with the MagNa Pure LC (Roche
150 Diagnostics AG, Rotkreuz, Switzerland) using the MagNa Pure LC TNA Isolation Kit
151 (Roche Diagnostics AG). RNA from tissue samples was extracted with the Qiagen

RNeasy® mini Kit (Qiagen, Hombrechtikon, Switzerland). In each batch of extractions, a negative control was used to monitor for cross-contamination. Extracted nucleic acids were stored at -20°C until PCR analysis.

Histology, immunohistochemistry and transmission electron microscopy. Cases 6 and 11 were examined post mortem and histology, immunohistochemistry (IHC) for feline herpesvirus-1 (FHV-1, performed in cases 6 and 11) and feline/canine parvovirus (performed in case 11) and transmission electron microscopy (TEM, performed in case 6) were conducted at the Institute of Veterinary Pathology, University of Zurich, Switzerland. The IHC for FCV (performed in case 11) was conducted by the Veterinary Laboratory Services, University of Liverpool, England. Samples for histological examination were collected from several skin locations, lung, liver, kidney, heart, pancreas and spleen; from case 11 samples were also collected from the oral mucosae, gut and mesenteric lymph node. All tissue samples were fixed in 4% neutral buffered formalin for 24 h, routinely processed for paraffin embedding, sectioned to prepare 2 - 3 µm thin sections and stained with hematoxylin and eosin (HE) and Periodic acid Schiff (PAS). The IHC for FCV was conducted on sections of skin, liver, spleen, lung, kidney and pancreas from case 11 according to published methods (Coyne et al., 2006b). Skin samples from cases 6 and 11 were examined immunohistochemically for FHV-1 according to published methods (Suchy et al., 2000). The IHC for feline/canine parvovirus was performed on gut samples from case 11 using a monoclonal anti feline/canine parvovirus antibody (MC2064, AbD Serotec, Puchheim, Germany). For TEM, tissue was dewaxed and refixed in 2.5% glutaraldehyde followed by osmium tetroxide fixation and processed using routine protocols.

Hematology and blood biochemistry. Hematology and blood biochemistry were performed in cases 1 to 6 and 11 at the Clinical Laboratory, Vetsuisse Faculty,

University of Zurich, on a Sysmex XT-2000iV (Sysmex Corporation, Kobe, Japan) (Weissenbacher et al., 2011) and Cobas Integra 800 instrument (Roche Diagnostics AG). The laboratory's own reference intervals were used for the adult cats and published reference intervals were used for the kittens (Meyers-Wallen et al., 1984).

Virus isolation, titration and neutralization. For virus isolation, samples were filtered (Filtropur S 0.45 µm syringe filter, Sarstedt, Nümbrecht, Germany), incubated on 80% confluent Crandell-Rees feline kidney (CRFK) cells on 24-well plates (TPP Tissue Culture Testplate 24, TPP Techno Plastic Products AG, Trasadingen Switzerland) and cultured using RPMI 1640 Medium (Sigma-Aldrich) supplemented with 10% heat inactivated fetal calf serum (FCS, Invitrogen, Basel, Switzerland), 2 mM L-glutamine (Gibco, Life Technologies) and 1x antibiotic-antimycotic (Gibco, Life Technologies). For each sample culture, a negative medium-only control was run in parallel. The samples were incubated on cells for two hours before 300 µl of complete medium were added. The cells were fed daily and evaluated for the presence of a cytopathic effect (CPE). If either a CPE was visible, or after a maximum of 7 days, the supernatant was collected for TNA extraction and stored at -80°C until required for virus neutralization. Prior to virus neutralization, the FCV isolates were expanded using 2 x 10^5 cells/ml of feline embryo A (FEA) cells (Jarrett et al., 1973). Virus neutralization assays were performed on the isolates using the method described previously (Addie et al., 2008). Virus isolates were tested for neutralization against a panel of eight antisera raised in four pairs of cats infected once by the oronasal route with 1 ml of a viral suspension containing 10^6 $TCID_{50}$ of FCV-F9, FCV-255, FCV-G1 or FCV-431 (one pair of cats was infected with each strain). The end point was calculated as the reciprocal of the highest serum dilution that showed CPE in no more than two of four wells. Similarly, the homologous titers of the antisera were calculated by testing them for neutralization against the relevant

FCV vaccine strain (FCV-F9, FCV-255, FCV-431 or FCV-G1).

Diagnostic assays. For FCV testing, a previously described real-time TaqMan reverse transcription (RT)- quantitative (q)PCR assay was used (Helps et al., 2002). The assay was optimized prior to the start of the experiment. The reaction contained 1 x One step RT-qPCR MasterMix Low ROX (Eurogentec, Seraing, Belgium), 300 nM forward primer, 900 nM reverse primer, 250 nM probe, 5 µl nuclease-free water (Gibco, Life Technologies) and 0.125 µl Euroscript (Eurogentec). The temperature profile was 30 min at 48°C, followed by 10 min at 95°C and 45 cycles of 15 sec at 95°C, followed by 1 min at 60°C. For the detection of FHV-1, a published real-time qPCR assay was used (Vogtlin et al., 2002). For feline leukemia virus (FeLV) and feline immunodeficiency virus (FIV) viral RNA detection in oropharyngeal cytobrushes (cases 7 to 10), previously described RT-qPCR assays were applied (Klein et al., 2001; Tandon et al., 2005). The real-time qPCR reactions were run on an ABI 7500Fast Real-Time PCR system (Applied Biosystems, Rotkreuz, Switzerland). Positive and negative controls were run with each assay. For FeLV p27 antigen and FIV antibody detection, a published ELISA (cases 1 to 5 and 11) (Calzolari et al., 1995; Lutz et al., 1983) or a commercially available Snap Test (case 7, FIV & FeLV Combi, Labor Laupeneck, Bern, Switzerland) was used.

Capsid gene amplification and sequencing. The capsid (VP1) gene from a total of 18 FCV isolates was sequenced using previously published primers (Primers AoA and AoS) that amplify 1945 nucleotides of the ORF 2 of FCV (Ohe et al., 2006). The FCV isolates derived from the eleven symptomatic cats (cases 1 to 11, Table 1), four in-contact cats in shelter 1 (queen and in-contact cats 1 to 3, Table 1) and three cats not in contact with symptomatic cats in shelter 1 (non-contact cats 1 to 3). RT-PCR and PCR amplification was performed with the SuperScript® III One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase (Invitrogen) using standard cycling

conditions. PCR products were separated on a 1.5% agarose gel, and amplicons of the appropriate size were cut and purified using the GenElute^{TM} Gel Extraction Kit (Sigma-Aldrich). Direct sequencing of the purified amplicons was performed with the amplification primers (AoA, AoS) (Ohe et al., 2006) and with published (P1, P2) (Coyne et al., 2007) or newly designed internal primers (S_FCV_FL.829f: 5'-CTA TCA CCT GAT GTC TGA TAC TGA - 3'; S_FCV_FL.1243r: 5'-CAC AAT AGA GTC GGT GGC AAT TCC A-3'; S_FCV_FL.1265r: 5'-GCC AAC CAT CAG GTA TGC CT-3'; S_FCV_La.543f: 5'-GCT TGG TCT GGM TCT ATT GA- 3'; FCVSeq_6145_6164f: 5'-CAY YTD ATG TCT GAY ACT GA-3'; FCVSeq_6705_6725r: 5'-GGR ATK GTD GTR TCD GGC CA-3') at a commercial laboratory (Microsynth, Balgach, Switzerland) under standard conditions.

Phylogenetic analyses. Nucleotide sequence editing, assembly and alignment were done using Geneious Version 7.1.7. Only the nucleotides available for all included sequences (1616 bp of the ORF2 of the capsid VP1 gene) were used to calculate nucleotide identities and for phylogenetic analyses. Amino acids were aligned using CLUSTAL W and BLOSUM cost matrix (Henikoff and Henikoff, 1992). The alignment was cut to 75 and 71 amino acids corresponding to residues 391 - 465 and 480 - 550, respectively, on ORF2 for the reference sequence FCV-F9. Phylogenetic analysis was performed using CLUSTAL W (Thompson et al., 1994) and MEGA version 6. A phylogenetic tree was created by the Neighbor-Joining algorithm, using a distance matrix corrected for nucleotide substitutions based on the Tamura-Nei model and for amino acid substitution using the Poisson model. The dataset was re-sampled 1,000 times to generate bootstrap values.

Nucleotide sequence accession numbers. Nucleotide sequences have been submitted to GenBank under accession numbers KP862861 to KP 862878.

Results

The eleven cases occurred between November 2011 and April 2014 in two shelters and two veterinary clinics in four geographically distinct locations in the two adjacent countries Switzerland and Liechtenstein.

Cases 1 to 5

Cases 1 to 5 occurred in November 2011 in shelter 1 in Schaan, Liechtenstein; these were five non-vaccinated three-month-old domestic shorthair (DSH) littermates. The kittens had been brought to the shelter two months before clinical signs occurred and were housed in a cage together with the queen (DSH, not vaccinated, age unknown). Three cats (in-contact cats 1 to 3, DSH, not vaccinated, 3 months, 4 months and 3 years old, respectively) were located in a neighboring cage separated by a mesh; nose-to-nose contact could occur between the three in-contact cats and the symptomatic kittens. In-contact cat 2 entered the shelter 17 days before the first signs occurred in cases 1 to 5. The affected kittens showed apathy, anorexia, fever, salivation, edema of the paws and pinna, tongue ulcerations and skin ulcerations around the head and paws. The queen and the three in-contact cats showed no clinical signs. Cases 1 to 5 showed moderate anemia, lymphopenia and leukopenia, with a left shift in one kitten (data not shown). All affected kittens tested FCV-positive but were negative for FHV-1, FeLV and FIV (Table 1). FCV was detected in the queen and the three in-contact cats (Table 1) and in 7 of 27 cats (26%) kept outside the quarantine room in shelter 1; six of these latter cats were clinically healthy and one showed signs of URTD. Cases 1 to 5 were treated symptomatically with antibiotics (chloramphenicol and cefovecin) and non-steroidal anti-inflammatory drugs (meloxicam) and recovered within 10 days.

Case 6

Case 6 was a vaccinated 10-year-old male castrated DSH cat without outdoor access that lived together with two other cats. Case 6 was brought into the small animal clinic of the Vetsuisse Faculty of the University of Zurich, Switzerland (clinic 1) in July of 2012 because of obstructive feline lower urinary tract disease. After unsuccessful conservative treatment, the cat received a perineal urethrostomy and was discharged with antibiotic (amoxicillin-clavulanic acid) and anti-inflammatory treatment (meloxicam). One day after discharge, the cat was presented again with apathy, fever and swollen paws. Within two days, the cat developed head oedema, tongue ulceration, skin pustules and ulcerations at the belly and around the anus (Supplementary Figure 1). The cat showed severe lymphopenia, left shift, moderate to severe hyperbilirubinemia, moderate hypoproteinemia, hypoalbuminemia and hyponatremia and slight hyperglycemia (data not shown). The cat tested FCV-positive in the oropharyngeal cytobrush, as well as in blood, edema and pustule fluid, but tested negative for FHV-1 (Table 1). Despite symptomatic treatment with intravenous infusions, antibiotics (amoxicillin-clavulanic acid), pain medication (buprenorphine), anti-inflammatory drugs (meloxicam), antiemetics (ondansetron) and a proton pump inhibitor (esomeprazole), the clinical condition of the cat deteriorated. The cat was euthanized four days after the second presentation. At necropsy, the cat was icteric and had marked subcutaneous edema on the head and paws. Skin histology revealed prominent intraepidermal and suprabasal pustules filled with numerous degenerated neutrophils (Figure 1 a). The lesions extended to full-thickness necrosis of the epidermis or extended into the dermis, obscuring the dermal-epidermal junction. The hair follicular epithelium was also involved in the necrotizing process. In the liver, the hepatocytes showed fading nuclei and yellow intracytoplasmic pigmentation; some bile duct capillaries were congested by bile

plugs (intrahepatic cholestasis). There was a mild nonspecific periportal infiltration with neutrophils, lymphocytes and plasma cells. The hepatocytes were dissociated due to autolysis, but there was no evidence of necrosis or vasculitis. Pancreas and spleen were unremarkable. The evaluation of the intestine was reduced due to autolysis but a few crypt abscesses could be found.

In TEM, intracytoplasmic paracrystalline virion arrays were detectable in a follicular adnexal epithelial cell (data not shown). IHC for FHV-1 in skin lesions was negative and IHC for FCV was not performed.

Cases 7 and 8

Cases 7 and 8 occurred in August 2012 in shelter 2 in Lausanne, Switzerland and involved four non-vaccinated two-month-old DSH kittens from one litter. The kittens had entered the shelter as newborn kittens two months before the first clinical signs occurred. Samples were collected from two kittens (cases 7 and 8) for the present study (Table 1). The kittens displayed apathy, fever, nasal discharge, and ulcers on the skin (muzzle, pinna and paws) and tongue. The kittens tested FCV-positive and negative for FHV-1, FeLV and FIV (Table 1). After symptomatic treatment with antibiotics (amoxicillin-clavulanic acid) and a non-steroidal anti-inflammatory drug (metamizole), the cats recovered within four to five days.

Cases 9 and 10

Cases 9 and 10 occurred five months later again in shelter 2 and involved a litter of three three-month-old DSH kittens that was born in the shelter. Samples were collected from two kittens (cases 9 and 10) for the present study (Table 1). The three kittens initially showed signs of apathy, anorexia and diarrhea, but recovered with symptomatic treatment with antibiotics and a spasmolytic drug. Three weeks after the first occurrence of clinical signs, the kittens were vaccinated against panleukopenia,

FCV and FHV-1 (Feligen®, Virbac, Glattbrugg, Switzerland). Two days later, one of the kittens was found dead. No samples were available from this animal. Ten days later, the other two kittens developed severe apathy, skin and lip ulceration, and edema of the pinna and front legs. Both kittens tested FCV-positive and one kitten tested positive for FIV-viremia (Table 1). Both cats were negative for FHV-1 and FeLV (Table 1). Despite symptomatic treatment with antibiotics (amoxicillin-clavulanic acid) and a non-steroidal anti-inflammatory drug (meloxicam), the clinical condition of the two kittens deteriorated and both cats developed dyspnea. Radiographic examination of the thorax of one of the kittens showed a generalized, severe interstitial to alveolar lung pattern with consolidation of the ventral lung lobes. Radiographs were compatible with pneumonia. One kitten died and the other was euthanized. No necropsies were performed.

Case 11

A vaccinated 10-year-old, male castrated DSH cat, kept as single cat with outdoor access, was brought into a small animal clinic in Oftrigen-Zofingen, Switzerland (clinic 2) in April 2014 with fever and a swollen paw. The cat received antibiotic (amoxicillin-clavulanic acid) and anti-inflammatory treatment (meloxicam) and was sent home. Two days later, the cat was presented again with fever and head and paw edema. Within three days, the cat developed generalized edema and severe jaundice. The cat showed a left shift and moderate lymphopenia, severe hyperbilirubinemia, hypoproteinemia and hypoalbuminemia, moderate hyponatremia and slight hyperglycemia. The cat tested FCV-positive in mucosal swabs and blood, and subsequently in biopsies of oral mucosa, skin and liver that were obtained post mortem (Table 1). Tests were negative for FHV-1, FeLV and FIV (Table 1). The antibiotic treatment was changed to fluoroquinolones and clindamycin and the cat

was treated symptomatically with intravenous infusions and an anti-inflammatory drug (meloxicam). Because the clinical condition continued to deteriorate, the cat was euthanized four days later. At necropsy, the cat was icteric and showed subcutaneous edema mainly on the fore limbs, around the knees, on the head, thorax and back. No ulcerative or pustular lesions were visible on the oral mucosa, the paws and on the haired skin. In skin histology, case 11 showed different degrees of epithelial necrosis, but the stratum corneum remained intact (Figure 1b). Liver histology revealed mild lipidosis and a slightly increased amount of lipidgranulomas; the yellow intrahepatocytic pigment could be identified as hemosiderin by Prussian blue staining. No histological lesions could be found in pancreas or spleen. There was mild peripancreatic fatty tissue necrosis that was interpreted as an artifact because of a lack of cellular reaction within the pancreas. The cat showed acute crypt cell necrosis in the jejunum and acute multifocal purulent and necrotizing colitis. The necrotizing colitis was associated to fungal infection with morphology of Aspergillus species. Fungal hyphae were not found in any other tissue of the cat. The IHC for feline/canine parvovirus performed on gut samples of case 11 was negative. The IHC for FCV demonstrated clusters of FCV-positive basal cells adjacent to degenerated cells and some FCV-positive cells in the liver and spleen (data not shown). The IHC for FHV-1 in skin lesions was negative.

Genetic and phylogenetic analyses of FCV isolates

From a total of 18 FCV isolates, 1,616 nucleotides of the ORF 2 encoding the capsid (VP1) protein of FCV were sequenced and phylogenetically analyzed (Figure 2). The FCV isolates from the different disease outbreaks were different from each other (74.3 - 82.6% nucleotide identity) as well as from published FCV isolates from VSD outbreaks (74.7 - 79.3% nucleotide identity when compared with FCV AY560117,

380 DQ91079, EU202915 and DQ910795).

381 The FCV isolates from shelter 1 (from cases 1 to 5, from the queen and the three in-
382 contact cats) shared 99.5 - 99.9% nucleotide identity in the capsid (VP1) gene. The
383 capsid (VP1) gene from the FCV isolates from cats kept outside the quarantine room
384 in shelter 1 (non-contact cats 1 to 3) showed only moderate sequence identity (83.5 -
385 84.0% nucleotide identity) with isolates from the affected kittens (cases 1 to 5), the
386 queen and the three in-contact cats. Phylogenetic analysis revealed that the FCV
387 isolates from the non-contact cats were phylogenetically related to but distinct from
388 the isolates of the affected kittens (Figure 2).

389 The FCV isolates from the two disease outbreaks that occurred in shelter 2 five
390 months apart in two different litters (cases 7 to 8 and 9 to 10, respectively) appeared
391 to be distinct (82.5 - 82.6% nucleotide identity, Figure 2). Of note, the isolates from
392 cases 9 and 10 were clearly distinct from the vaccine strain FCV-F9 (M86379, 77.3 -
393 77.4% nucleotide identity, Figure 2); FCV-F9 was the vaccine strain that these cats
394 had received prior to the onset of disease. The FCV isolates from cases 6 and 11
395 that occurred in clinic 1 and 2, respectively, were phylogenetically distinct from the
396 other isolates in this study, as well as from published FCV isolates from VSD
397 outbreaks (Figure 2).

398 Comparative analysis of the amino acid sequences of the capsid VP1 region of the
399 15 isolates did not reveal consistent substitutions in all FCV isolates of this study
400 (Figure 3 a and b). The majority of substitutions clustered to region D and
401 hypervariable region E of the capsid VP1 (Figure 3). Several amino acid changes
402 were observed in residues known either to be associated with the selection of
403 neutralization resistant virus mutants or to be part of linear B-cell epitopes (Figure 3 a
404 and b)(Radford et al., 1999; Tohya et al., 1997). Some of the amino acid substitutions
405 recently reported to be associated with VSD were also observed in the FCV isolates

406 from this study (V430T, N443S, G450D, D452E and V456M; Figure 3 a). Some of
407 these substitutions were also present in published sequences from FCV isolates not
408 associated with VSD (V430T, N443S, D452E; Figure 3 a). Other published
409 substitutions were not evident in any of the FCV isolates in this study (E399K,
410 T438V, A448K, D455M, K458S), and some residues displayed heterogeneous
411 substitutions (E399K, A448K, G450D, D455M; Figure 3 a).
412 When the amino acid sequences of the capsid VP 1 region were compared between
413 the FCV isolates of the affected litter (cases 1 - 5), the FCV isolates of the queen and
414 the three in-contact cats and the FCV isolates of cats with no contact with the
415 affected litter in shelter 1 (non-contact cats 1 - 3), a total of 34 substitutions were
416 found that were present in the isolates of the affected kittens and healthy in-contact
417 cats and absent in the FCV isolates of the non-contact cats (Figure 3 b, and I101V,
418 N120S, S128G, Q202D, A303T, S318A, K575R, I615V). Again, most substitutions
419 clustered to region D and hypervariable region E of the capsid VP1 gene (Figure 3
420 b).

421 **Virus neutralization of FCV isolates**

422 Thirteen FCV isolates from this study were tested by virus neutralization against eight
423 antisera recognizing the common FCV vaccine strains (FCV-G1, FCV-431, FCV-255
424 and FCV-F9, Table 2); no viruses were available for virus neutralization from case 2
425 and the queen in shelter 1. FCV isolates from the same disease outbreak showed
426 similar neutralization patterns, whereas virus neutralization patterns were clearly
427 distinct between different disease outbreaks. FCV isolates from cases 6, 9 and 10
428 showed low neutralization titers with all antisera tested. The homologous
429 neutralization titers of the antisera S7 and S8 (FCV-F9) were three to nine times
430 lower than the homologous titers of the antisera S1 to S6 (FCV-G1, FCV-431 and

FCV-255). The low to undetectable neutralization titers obtained with antisera S7 and S8 (FCV-F9) for the FCV isolates tested here could therefore be related to the lower potency of these antisera. Antisera raised against the same FCV vaccine strain in two different cats showed marked differences in the neutralization titers for the same FCV isolate (S1 and S2, S3 and S4, S5 and S6, respectively; Table 2).

Discussion

The present case series provides a clinical, histological and genetic characterization and analysis of virus neutralization patterns of severe, non-epizootic forms of FCV infections associated with head, paw or generalized edema and ulcerations on the head and limbs. The present cases had initial clinical presentations suspicious of VSD, but they lacked some characteristics that define the syndrome: namely some were missing inner organ involvement and high mortality and all were lacking epizootic disease spread (Radford et al., 2009).

The clinical presentation and disease course in cases 1 to 5 in shelter 1 and cases 7 to 8 in shelter 2 resembled the 'paw and mouth disease' syndrome described by Cooper and Sabine in 1972 (Cooper and Sabine, 1972). These kittens showed edema and/or skin ulcerations localized to the head and paws, but no signs of inner organ involvement or a systemic inflammatory response syndrome; all animals survived with supportive care. In contrast, the two adult cats (cases 6 and 11) and the kittens of the second outbreak in shelter 2 (cases 9 and 10) showed signs of a systemic inflammatory response syndrome and inner organ involvement, i.e. severe edema, left shift, icterus, hypoproteinemia (cases 6 and 11), dyspnea with radiographic signs of pneumonia (cases 9 and 10), intestinal crypt lesions (case 11) and the detection of FCV by IHC in the liver and spleen (case 11). These cases deteriorated quickly and died or were euthanized. Although the clinical presentations

and disease course in these cats resembled VSD, co-morbidities could have accounted for the severe outcome: case 6 had a prehistory of obstructive FLUTD and perineal urethrostomy, case 10 tested positive for FIV viremia, cases 9 and 10 had previously histories of diarrhea and case 11 showed signs of an intestinal fungal infection at necropsy. Furthermore, pancreatitis, pancreatic or hepatic necrosis, interstitial pneumonia or disseminated thrombosis which have been reported in cats with VSD (Hurley et al., 2004; Pedersen et al., 2000; Pesavento et al., 2004; Schorr-Evans et al., 2003) were not found during the necropsies of cases 6 and 11, although the histological evaluation was hampered by euthanasia and some degree of autolysis. Intestinal crypt necrosis, as found in case 11, was described in cases of VSD (Pedersen et al., 2000; Schulz et al., 2011), but some of these cats were co-infected with feline parvovirus. Case 11 tested negative for feline/canine parvovirus by IHC, but a fungal infection with morphology of Aspergillus species was detected in the colon; perhaps a consequence of the intense antibiotic therapy or terminal severe FCV infection with debilitation of the immune system (Pedersen et al., 2000). The present study suggests that severe forms of FCV infections can initially present similar to VSD, but high mortality and inner organ involvement is not always present, and disease severity might also depend on the immune status of the cat and aggravating factors, such as co-morbidities and crowding.

Remarkably, one severely affected cat in the present study (case 11) showed generalized edema and icterus, but no macroscopic skin lesions or oral ulcerations up until the time of death. The lack of cutaneous or oral lesions in this cat was in accordance with the histological findings, which revealed an intact stratum corneum overlying marked epidermal degeneration. Hence FCV infection should be included in the differential diagnosis for any cat presenting with head, paw or generalized edema, even in the absence of macroscopic ulcerations of the skin or oral cavity.

Another defining criterion of VSD is epizootic disease spread. This was not noted in any of the present outbreaks. Whether this was due to the strict quarantine measures that had been implemented upon FCV diagnosis, or related to intrinsic properties of the FCV strains, remains unknown. There have been two recent reports of single cases of non-epizootic VSD (Battilani et al., 2013; Meyer et al., 2011). Battilani et al. described a FIV-positive cat with fever, oral ulceration, liver necrosis and multifocal hemorrhage (Battilani et al., 2013). Interestingly, the FCV strains isolated from the oropharyngeal cytobrush and internal organs of this cat showed only moderate sequence identity in the capsid (VP1) gene. The in-contact cats remained clinically healthy, but tested FCV-positive; the healthy in-contacts were infected with a genetically distinct FCV strain. In the study reported by Meyer et al., the affected cat showed subcutaneous edema and necrotizing dermatitis, but there was no necrosis of organs other than the skin and oral cavity and none of the other six cats in the same household developed disease despite close contact (Meyer et al., 2011); unfortunately, the in-contact cats were not tested for FCV infection.

Similar to outbreaks of VSD (Battilani et al., 2013; Coyne et al., 2006b; Hurley et al., 2004; Meyer et al., 2011; Pedersen et al., 2000; Reynolds et al., 2009; Schorr-Evans et al., 2003; Schulz et al., 2011), all but one of the disease outbreaks in this study (case 11) originated in multi-cat environments. The high genetic evolution of FCV and high levels of replication in large groups of cats provide the ideal conditions necessary for the emergence of highly virulent strains (Radford et al., 2009). Outbreaks of VSD often start with the introduction of cats from large rescue colonies into another multi-cat environment, such as a veterinary clinic or shelter (Radford et al., 2007). In the present cases, the origin of the infection was unknown. The kittens in shelter 1 (cases 1 to 5) were brought to the shelter two months before the first clinical signs occurred. However, one asymptomatic kitten in close contact with the

kittens (in-contact cat 2) and infected with a similar FCV isolate (> 99% nucleotide identity of the 1616 bp of ORF2) entered the shelter 17 days before the first signs in the affected kittens occurred. It could be speculated that this in-contact cat might have introduced the FCV infection to the kittens. Genetic and phylogenetic analyses of the FCV isolates from shelter 1 revealed that the isolates from cats kept outside the quarantine room (non-contact cats 1 to 3) were phylogenetically related to the isolates of the affected kittens (cases 1 to 5), the queen and the three non-affected in-contact cats. This suggests that the FCV isolate causing this severe disease manifestation in shelter 1 was not introduced; rather it had evolved *de novo* in the shelter environment. The fact that the in-contact cats in shelter 1 remained clinically healthy is remarkable, since two of the in-contact cats were young, unvaccinated kittens from a different litter. Age and immune status appear not to be the sole reason for the observed differences in susceptibility to FCV-induced disease.

The kittens in shelter 2 were either born in the shelter (cases 9 and 10) or entered the shelter as newborn kittens (cases 7 and 8) two months before the first clinical signs occurred. These animals might have acquired infection in the shelter environment. Phylogenetic analyses revealed that the FCV strains from the two outbreaks in shelter 2 were distinct, suggesting that they were not directly transferred between the outbreaks. The FCV infection of case 6 might represent a nosocomial infection that had been acquired during the first hospitalization in clinic 1, although no cases with similar clinical signs were reported in clinic 1 in the weeks before or after case 6 was diagnosed. The infection source remained unresolved for case 11. This animal already showed paw edema when the cat was presented to the veterinary clinic; a nosocomial transmission therefore seems unlikely. The cat originated from a single cat household but had free access to the outdoors.

So far, attempts to identify genetic markers unique to FCV strains that cause VSD

534 have been inconclusive (Abd-Eldaim et al., 2005; Foley et al., 2006; Prikhodko et al.,
535 2014; Rong et al., 2006). When we compared the amino acid sequences of the
536 capsid VP1 region of the FCV isolates from this study to each other, no characteristic
537 signatures could be identified. The genetic and phylogenetic analyses revealed that
538 the FCV isolates causing these severe forms of disease were similar to other FCV
539 strains and to VSD-associated strains. The isolates from different outbreaks were
540 phylogenetically unrelated and showed extensive genetic variability.
541 The kittens of one of the affected litters in shelter 2 (cases 9 and 10) had been
542 vaccinated against FCV between two and ten days prior to the development of FCV-
543 associated disease. The private veterinarian therefore suspected that the clinical
544 signs could have been caused by the vaccine strain. However, sequence analyses of
545 the FCV isolates from these animals showed only 77.3 - 77.4% nucleotide identity
546 with vaccine strain FCV-F9, which had been used to vaccinate the kittens. These
547 findings imply that the temporal relationship between vaccination and the
548 development of disease was a random coincidence, although vaccination might have
549 influenced the disease course if the cats had a pre-existing infection with FCV.

550 The four cats that died (cases 6 and 9 to 11) had been vaccinated against FCV, but
551 three of them had only been incompletely vaccinated. The two kittens that died
552 (cases 9 and 10) had received only one shot of FCV vaccine two days before the first
553 kitten of the litter died (not included in the study) and 10 days before the first
554 symptoms in kittens 9 and 10 occurred. One of the adult cats that died (case 6) had
555 last received a FCV vaccine 3 years previously. Results of virus neutralization assays
556 suggested that the cases in shelter 1 and clinic 2 and one of the outbreaks in shelter
557 2 might have been potentially prevented by vaccination with the optimal vaccine
558 strain. However, none of the antisera raised against four different vaccine strains

showed high cross-neutralization of all of the FCV isolates from this study, indicating that no single vaccine strain would have been predicted to protect against all outbreaks of disease reported in this study. Furthermore, antisera raised against the same FCV vaccine strain in different cats showed marked differences in the neutralization titers for the same FCV isolate, suggesting a remarkable individual variation in the immune response elicited to FCV. The fact that the antisera raised against the vaccine strain FCV-F9 showed low to undetectable neutralization titers with all FCV isolates from this study could be explained by the lower potency of the F9 antisera, as indicated by the low homologous antibody titers. Finally, serum neutralization might underestimate protection, since cell-mediated immune mechanisms are also thought to play a role in protection against FCV infection, particularly when modified live-virus vaccines are applied (Lesbros et al., 2013).

Conclusions

The present case series provides an extensive investigation of eleven cases of severe forms of FCV infections associated with edema and skin ulcerations. Most of the cases occurred in multi-cat environments and the cats presented with a spectrum of clinical signs and disease severity. The FCV isolates from the affected cats exhibited distinct genetic backgrounds and virus neutralization patterns. Disease severity appeared, on the one hand, to depend on intrinsic properties of the FCV isolate but, on the other hand, also on the susceptibility of the cats and on aggravating factors, such as co-morbidities or crowding. Our data suggest that severe forms of FCV infections can present initially with clinical signs similar to VSD, but high mortality and inner organ involvement is not always present and epizootic disease spread may be absent.

Competing interests

584 The authors declare that they have no conflicts of interest.

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775 Figure captions

776 ***Figure 1: Skin histology of case 6 and 11.*** *a. Haired skin of the thigh, case 6: intraepidermal and*
777 *suprabasal pustules (arrow) associated with full-thickness epidermal necrosis. Abrupt transition from*
778 *normal to affected skin. Many perifollicular degenerating neutrophils in the superficial dermis (HE). b.*
779 *Haired skin, paw, case 11. Segmental vacuolar degeneration of basal cells* to reticular degeneration*
780 *also of stratum spinosum und stratum granulosum cells** to full thickness necrosis*** with still intact*
781 *stratum corneum. Marked dermal edema with sparse inflammatory cells (HE).*

782 ***Figure 2: Phylogenetic analysis of 1,616 bp of ORF2 of FCV isolates of this study.*** *A total of 18*
783 *FCV isolates obtained within this study (in bold), 14 published FCV isolates reported in the USA,*
784 *Japan, France, the UK, Germany and Australia and the vaccine strain FCV-F9 are shown. FCV*
785 *isolates from VSD outbreaks are indicated with VS-FCV. Rabbit hemorrhagic fever virus (RHD) was*
786 *used as an outlier. GenBank accession numbers are shown in parentheses. The scale bar indicates*
787 *the number of estimated nucleotide substitutions per site. Only bootstrap values above 70% are*
788 *shown. The sequences derived from the following cases: case 6 (clinic 1), cases 7 to 10 (shelter 2),*
789 *case 11 (clinic 2), cases 1 to 5 (shelter 1); and from the queen and three in-contact cats, as well as*
790 *from three cats without contact with the affected kittens in shelter 1 (non-contact cats 1 to 3). TNA*
791 *extracted from oropharyngeal cytobrush was used for sequencing for all cats except for case 5, the*

queen and in-contact cats 1 and 2 for which TNA extracted from cell culture supernatant was used. In addition, for cases 6 and 11, TNA extracted from blood was used for sequencing; the sequences from oropharyngeal cytobrush and blood from the same cat showed > 99% sequence identity (data not shown).

Figure 3: Alignment of the capsid VP1 amino acid sequence of FCV isolates of this study. *a. Residues 391 to 465 of region D and hypervariable region E of the capsid VP1 protein of 15 FCV isolates obtained within this study (on top, shaded areas), of 4 FCV isolates associated with VSD (indicated with VS-FCV) and of nine other published FCV isolates are aligned to FCV-F9 (top sequence in the alignment). b. Residues 391 to 465 (top) and 480 to 550 (bottom) of region D and hypervariable region E of the capsid VP1 protein of the FCV isolates of cases, in-contact cats and non-contact cats in shelter 1 (shaded areas) and of 4 FCV isolates associated with VSD (indicated with VS-FCV) are aligned to FCV-F9 (top sequence in the alignment). GenBank accession numbers are shown in parentheses. Colored amino acids correspond to non-synonymous mutations in the RNA sequence compared to the FCV-F9 reference strain. Arrows indicate mutations previously described in FCV isolates associated with VSD, some of which were also observed in the FCV isolates from this study (V430T, cases 1 - 5 and in-contact cats, and cases 6, 9, 10 and 11 (Foley et al., 2006); N443S, cases 1 - 5 and in-contact cats (Abd-Eldaim et al., 2005); G450D, cases 7 and 8 (Prikhodko et al., 2014); D452E, cases 7,8 and 11 (Foley et al., 2006); V456M, case 6 (Prikhodko et al., 2014)). Asterisks indicate amino acid positions associated with selection of the neutralization-resistant virus mutants (Tohya et al., 1997) and the black bar marks a linear B-cell epitope mapped by Radford et al. (Radford et al., 1999). The "+" signs indicate the positions of the VP1 residues involved in putative contact between VP1 and fJAM-A (Bhella and Goodfellow, 2011). The triangles indicate amino acid substitutions present in all FCV isolates of the affected kittens, the queen and in-contact cats and absent in the FCV isolates of the non-contact cats in shelter 1. The three question marks in the sequence of case 2 at positions 441, 449 and 488 represent amino acid uncertainties K/N, N/T and T/I, respectively.*

Table

Table 1: Results for FCV, FHV-1, FeLV and FIV of symptomatic cats and of healthy in-contact cats. *Positive results are shown in bold.*

Location	Cat	Samples collected[1,2]	Date of sampling	FCV RT-qPCR	FHV-1 PCR[6]	FeLV[6]	FIV[6]
Shelter 1	**Case 1**	OC, blood	Nov 2011	**positive**[3]	negative	negative[7]	negative[9]
	Case 2	OC, blood	Nov 2011	**positive**[3]	negative	negative[7]	negative[9]
	Case 3	OC, blood	Nov 2011	**positive**[3]	negative	negative[7]	negative[9]
	Case 4	OC, blood	Nov 2011	**positive**[3]	negative	negative[7]	negative[9]
	Case 5	OC, blood	Nov 2011	**positive**[3]	negative	negative[7]	negative[9]
	Queen	OC	Nov 2011	**positive**	nt	nt	nt
	In-contact cat 1	OC	Nov 2011	**positive**	nt	nt	nt
	In-contact cat 2	OC	Nov 2011	**positive**	nt	nt	nt
	In-contact cat 3	OC	Nov 2011	**positive**	nt	nt	nt
Clinic 1	**Case 6**	OC, blood	Jul 2012	**positive**[3,4]	negative	nt	nt
		Edema and pustule fluid	Jul 2012	**positive**	nt	nt	nt
Shelter 2	**Case 7**	OC, blood	Aug 2012	**positive**[3]	nt	negative[7]	negative[9]
		OC/NS/CS	Oct 2012	**positive**	negative	negative[8]	negative[10]
	Case 8	OC/NS/CS	Oct 2012	**positive**	negative	negative[8]	negative[10]
Shelter 2	**Case 9**	OC/NS/CS	Jan 2013	**positive**	negative	negative[8]	negative[10]
	Case 10	OC/NS/CS	Jan 2013	**positive**	negative	negative[8]	**positive**[10]
Clinic 2	**Case 11**	OC/NS/CS, blood	April 2014	**positive**[4,5]	negative	negative[7]	negative[9]
		Mucosa, skin and liver	May 2014	**positive**	nt	nt	nt

[1] OC, oropharyngeal cytobrush, [2] OC/NS/CS, pooled material from oropharyngeal cytobrush, nasal and conjunctival swabs, [3] FCV RT-qPCR positive in the OC, [4] FCV RT-qPCR positive in blood, [5] FCV RT-qPCR positive in the OC/NS/CS, [6] nt, not tested; [7] result of FeLV ELISA from blood, [8] result of FeLV RT-qPCR from OC/NS/CS, [9] result of FIV ELISA from blood, [10] result of FIV RT-qPCR from OC/NS/CS (for details see Materials and Methods).

Table 2: Virus neutralization titers of FCV isolates from symptomatic cats and from healthy in-contact cats. *Maximal neutralization titers for each FCV isolate are shown in bold. Vaccination status and vaccine strain used in the cats are indicated. Homologous antibody titers of antisera S1 - S8 are shown at the bottom.*

Location	Cat	Vaccination status[2]	Vaccine strain	S1 [6] G1	S2 [6] G1	S3 [6] 431	S4 [6] 431	S5 [6] 255	S6 [6] 255	S7 [6] F9	S8 [6] F9
Shelter 1	**Case 1**	NV		<5	5	<5	15	5	**135**	<5	<5
	Case 3	NV		<5	5	<5	15	5	**405**	<5	<5
	Case 4	NV		<5	5	5	15	5	**405**	<5	<5
	Case 5	NV		<5	5	5	15	15	**135**	<5	<5
	In-contact cat 1	NV		<5	5	<5	15	15	**135**	<5	<5
	In-contact cat 2	NV		<5	5	<5	15	5	**405**	<5	<5
	In contact cat 3	NV		<5	5	<5	15	15	**405**	<5	<5
Clinic 1	**Case 6**	V [3]	FCV-F9	5	**45**	5	15	5	5	<5	15
Shelter 2	**Case 7**	NV		<5	**135**	15	**135**	<5	<5	<5	<5
	Case 8	NV		5	45	45	**135**	15	15	5	5
	Case 9	V[4]	FCV-F9	<5	**15**	5	<5	<5	5	<5	<5
	Case 10	V[4]	FCV-F9	<5	**15**	<15 [7]	5	<5	<5	<5	<5
Clinic 2	**Case 11**	V[5]	FCV-F9	<5	**405**	<15 [7]	45	15	135	<5	<5
Homologous antibody titres[1]				1215	1215	1215	3645	645	1215	405	405

[1] The homologous titers of the antisera were calculated by testing them for neutralization against the relevant FCV vaccine strain (FCV-F9, FCV-255, FCV-431 or FCV-G1), [2] V, vaccinated, NV, not vaccinated, [3] Case 6 was regularly vaccinated against FCV, FHV-1 and panleukopenia until 2009, [4] Cases 9 and 10 received one shot of a FCV, FHV-1, panleukopenia vaccine 10 days before the first symptoms of severe FCV infection occurred (see result section, cases 9 and 10), [5] Case 11 was vaccinated annually against FCV, FHV-1, panleukopenia and FeLV between 2007 -2013, [6] Virus neutralizing antibody titers of antisera S1 – S8 produced with FCV-G1 (S1, S2), FCV-431 (S3, S4), FCV-255 (S5, S6) and FCV-F9 (S7, S8), respectively, with the FCV strains isolated from each cat (for details see Material and Methods), [7] Neutralization titers <15 were not determined in these samples because of the limited volume of antiserum S3.

Figure 1

Figure 1 a

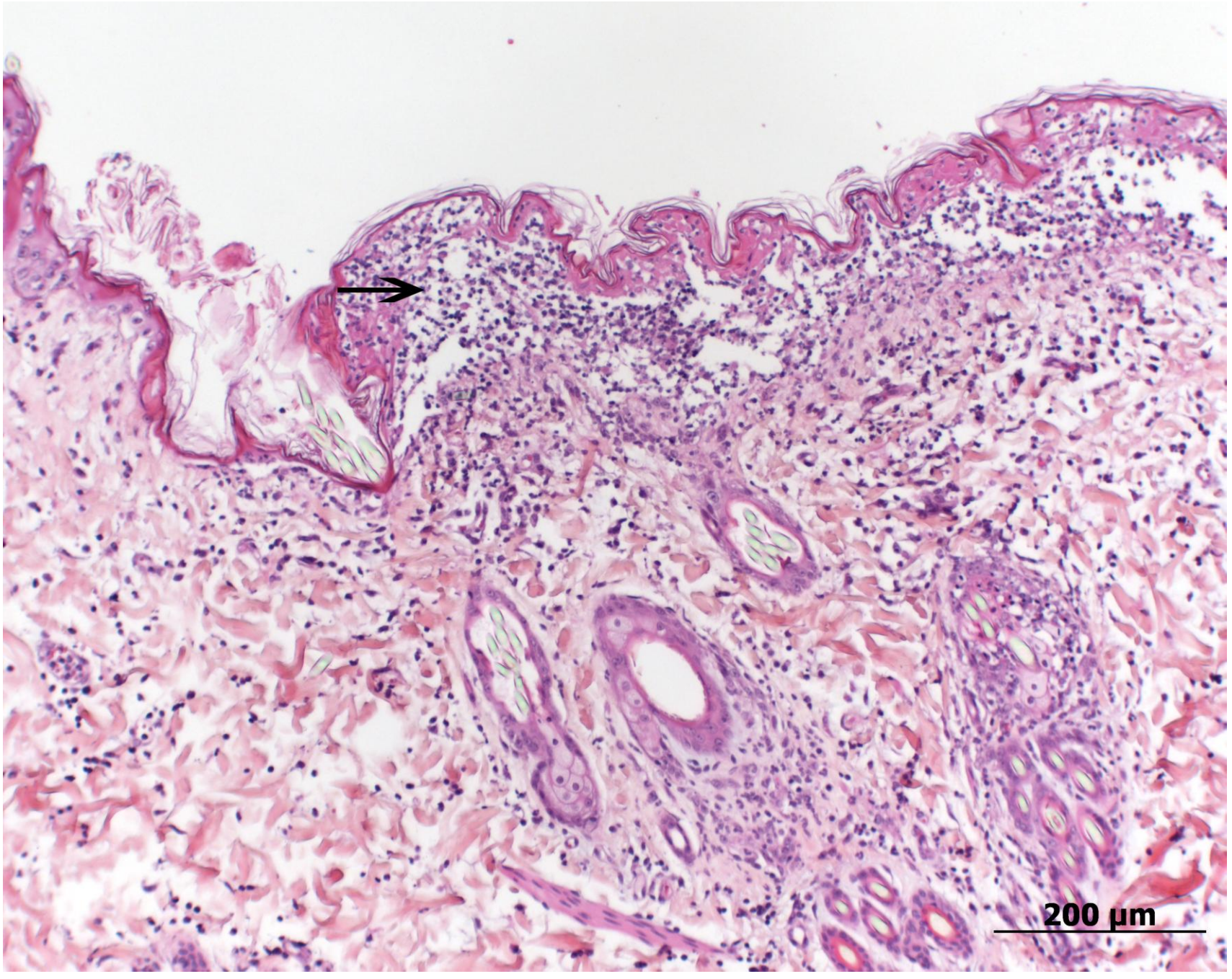


Figure 1 b

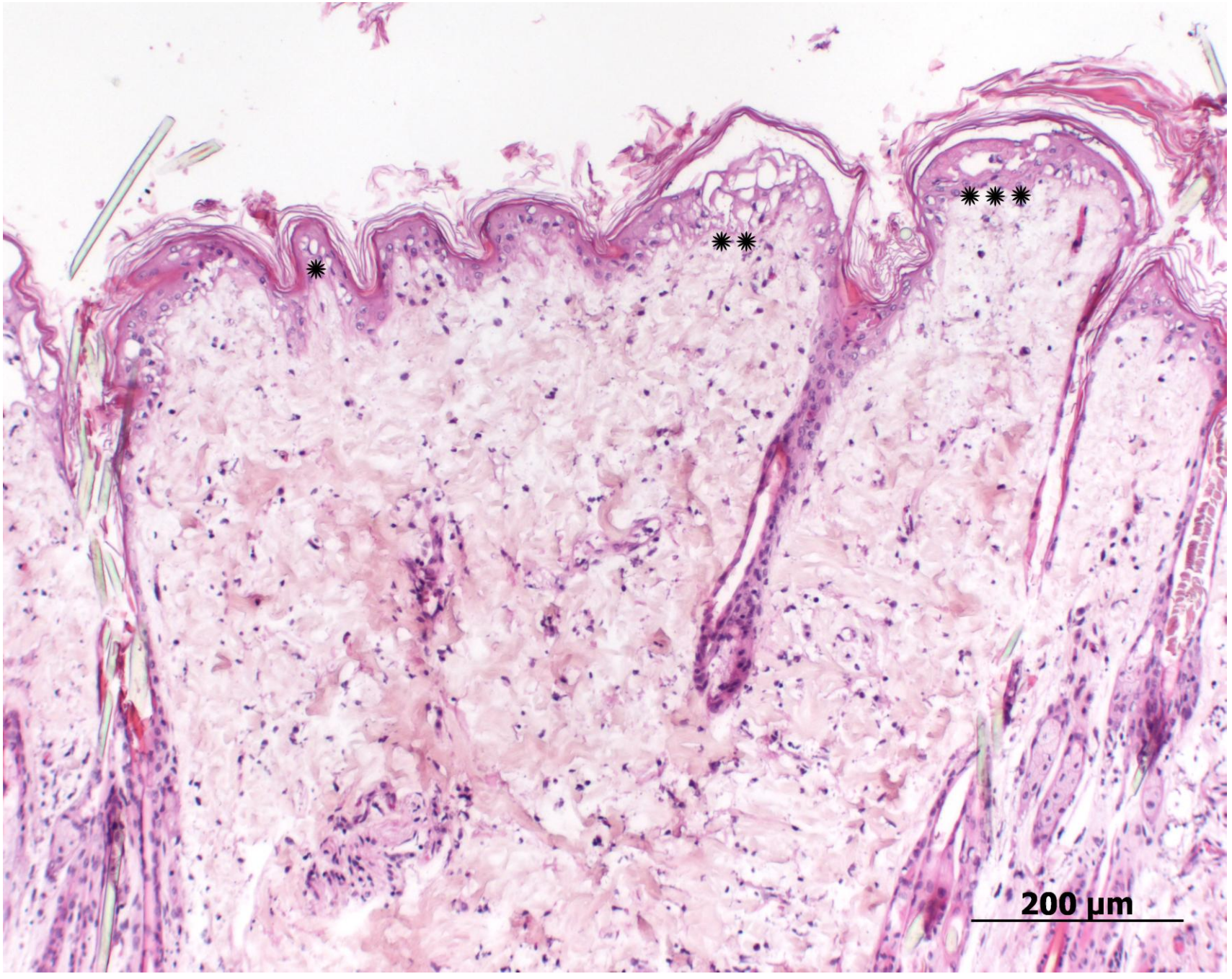


Figure 2

Fig. 2

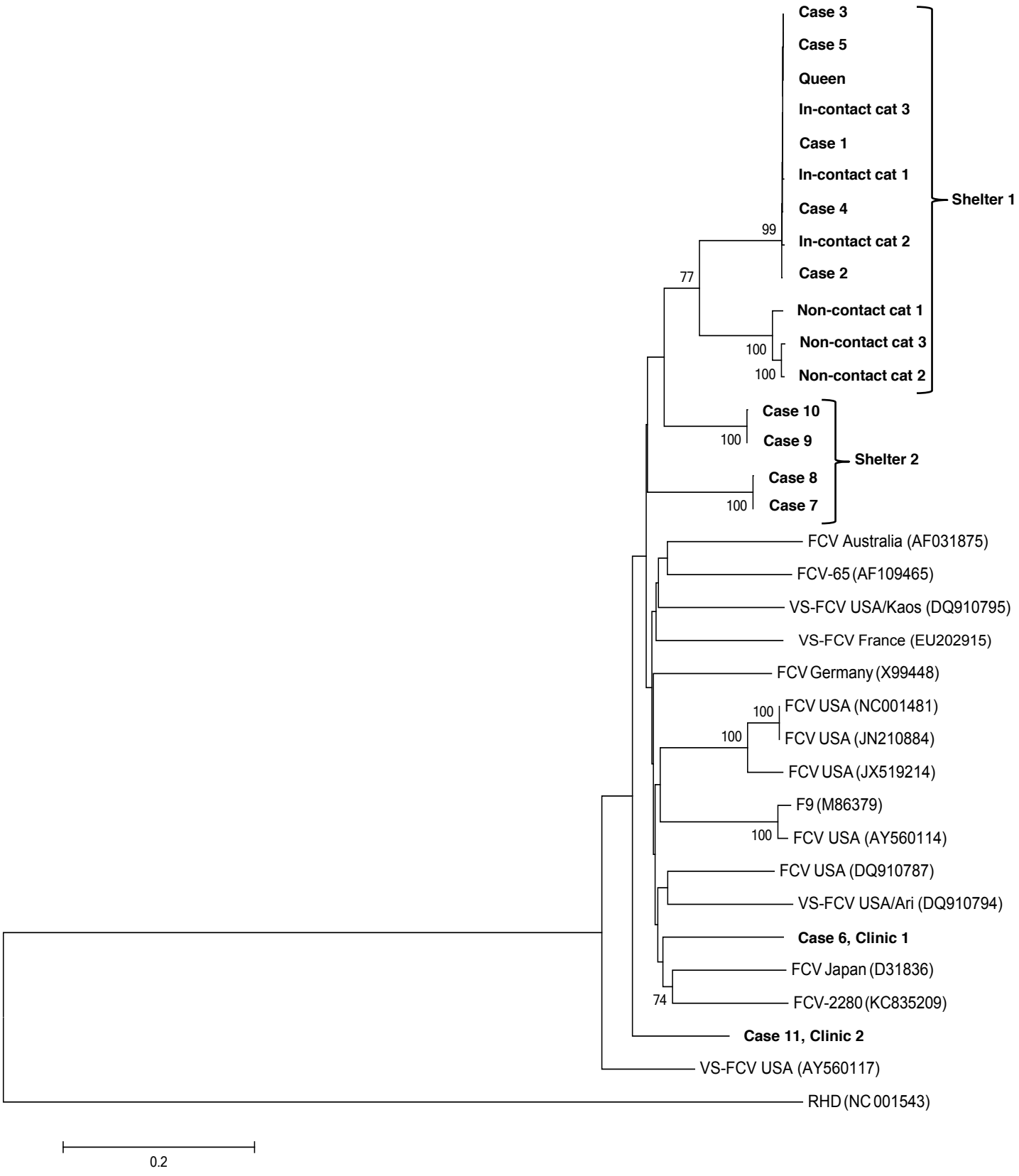


Figure 3

Figure 3 a

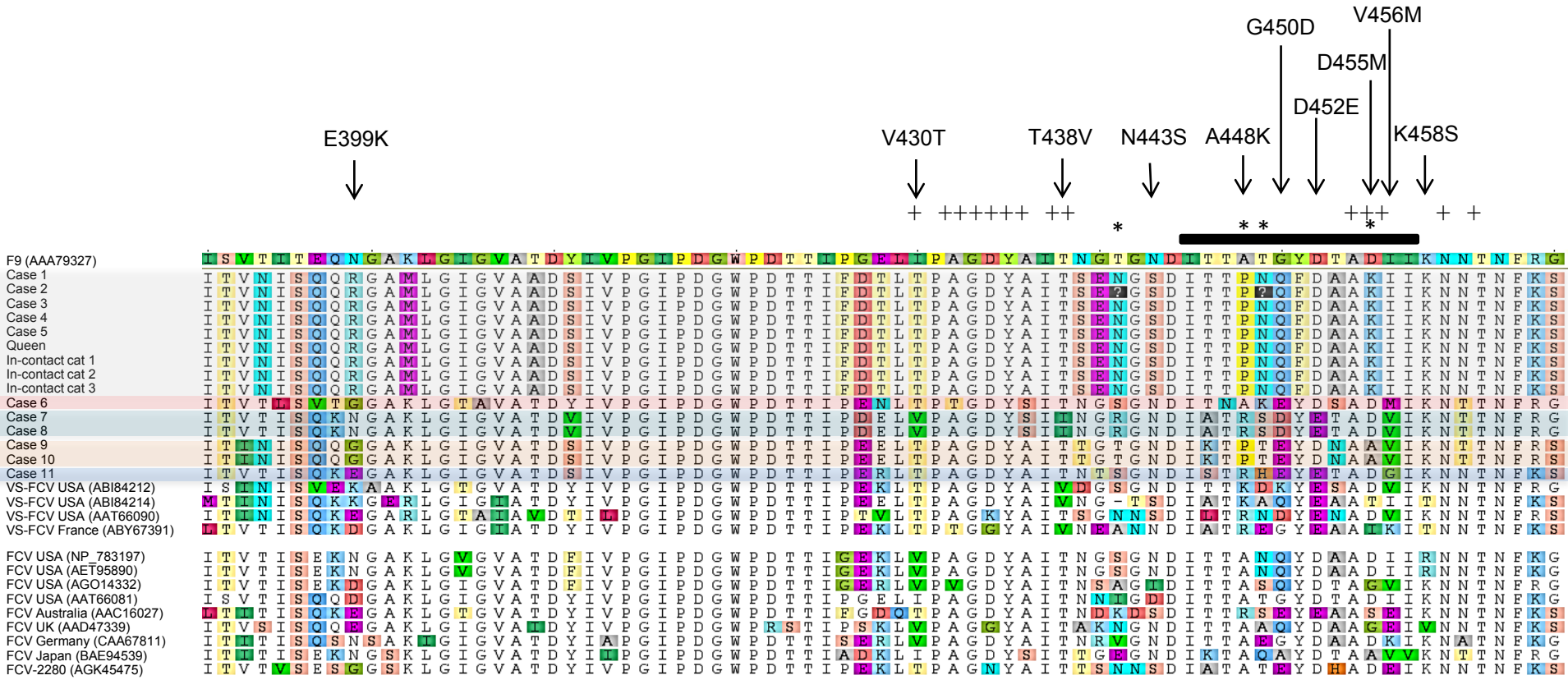
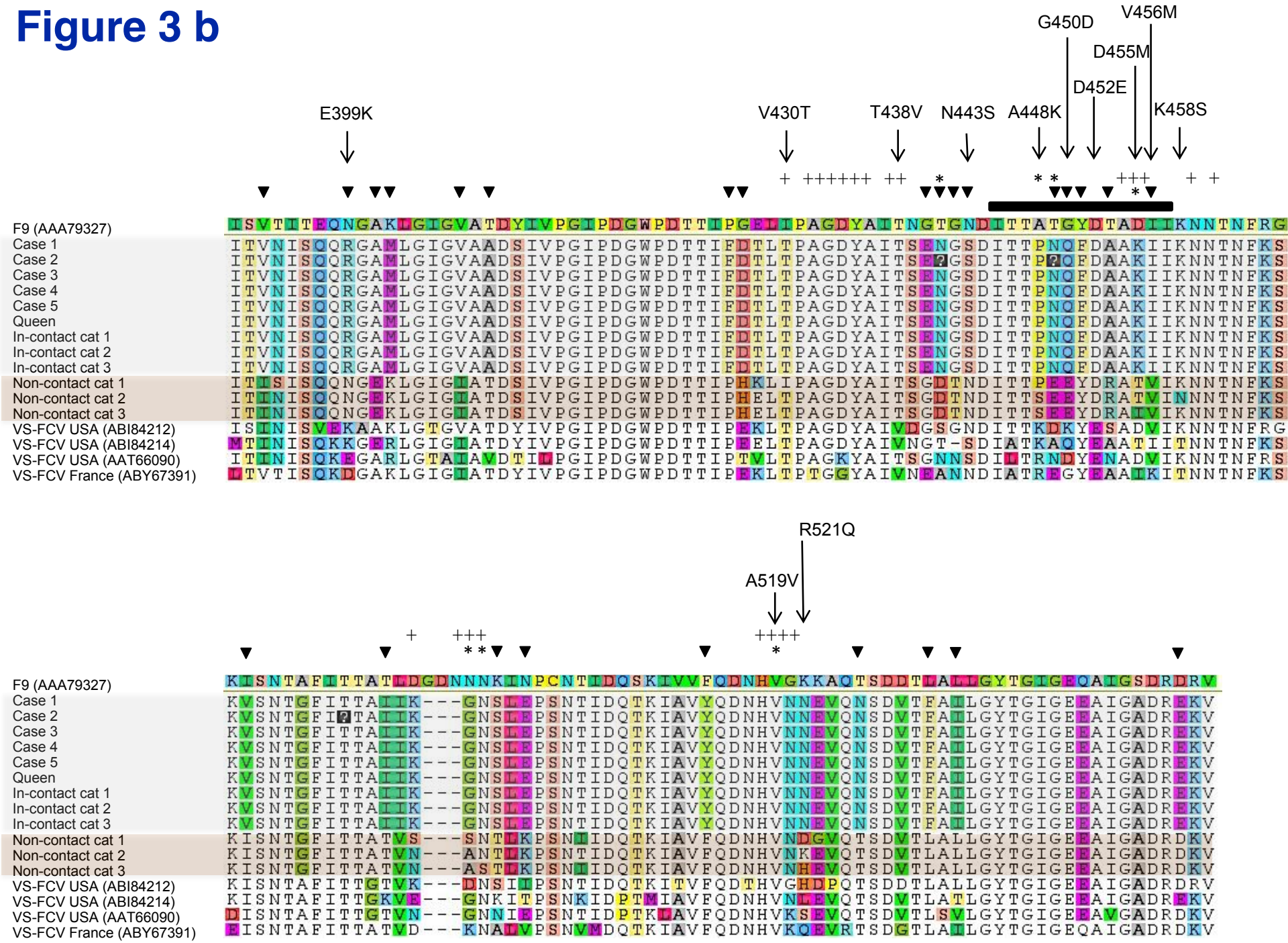
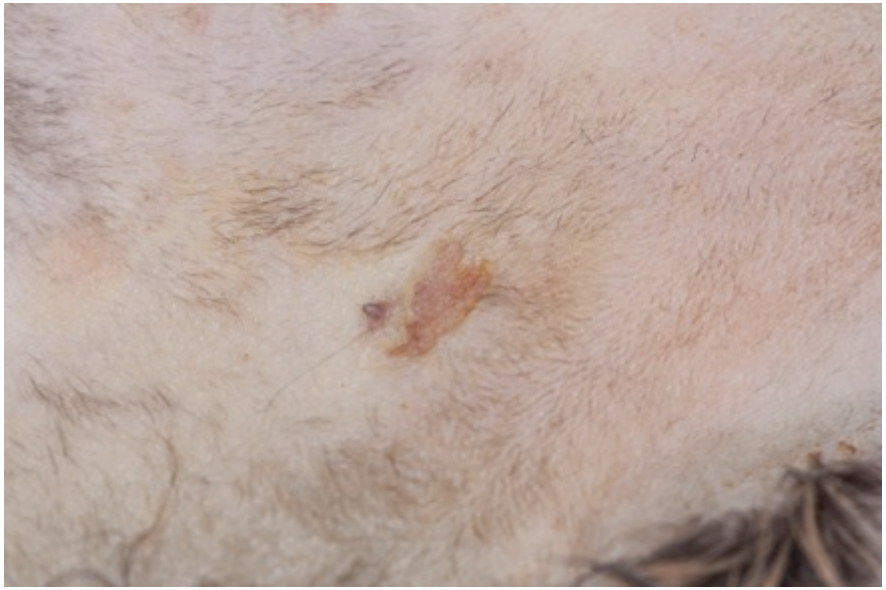


Figure 3 b

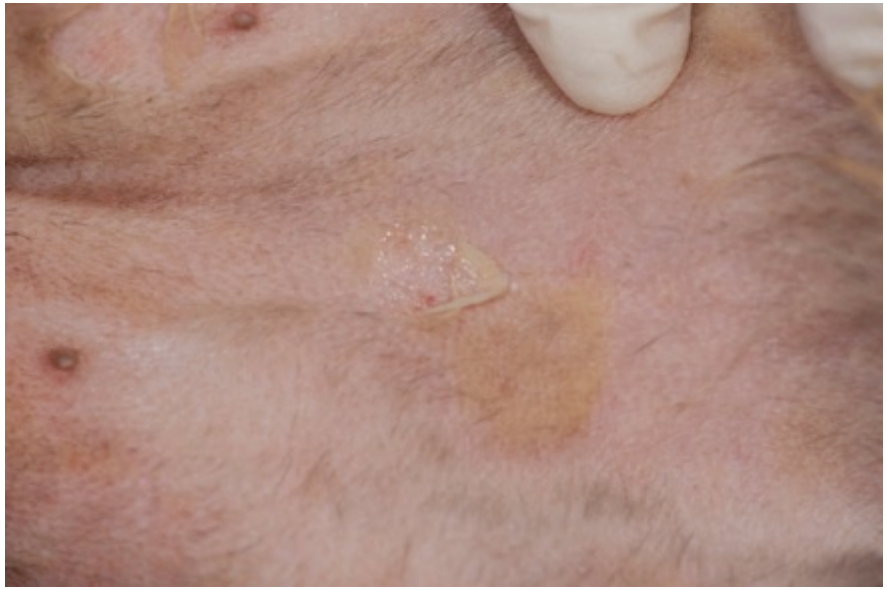

G450D
V456M
D455M
D452E
K458S
E399K
V430T
T438V
N443S
A448K
R521Q
A519V
F9 (AAA79327)
Case 1
Case 2
Case 3
Case 4
Case 5
Queen
In-contact cat 1
In-contact cat 2
In-contact cat 3
Non-contact cat 1
Non-contact cat 2
Non-contact cat 3
VS-FCV USA (ABI84212)
VS-FCV USA (ABI84214)
VS-FCV USA (AAT66090)
VS-FCV France (ABY67391)

Supplementary figure 1

a



b



c