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Seroepidemiological studies of coronavirus and influenza A virus infections in companion animals

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(met een samenvatting in het Nederlands)

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To my most beloved family

此书献给我最爱的家人

Table of contents

Chapter 1

General Introduction

Companion animals co-habit with humans and often play an important role in lives of many individuals and families [1,2]. The majority of households around the world have pets, and a large percentage of the human population has regular or occasional close contact with a variety of companion animal species. Very much like human to human contact, animal-human interaction also has inherent risks of pathogen transmission [3]. Even though the threat is not high in general, companion animal-associated zoonoses can occur with the consequences ranging from mild to mortal [3]. Typical examples of zoonotic diseases carried by companion animals are rabies, which mainly results in fatal consequences once symptoms appear, and toxoplasmosis that is associated with fetal death and miscarriage [4,5]. To date, companion animal zoonoses have received some attention, as a result of the close relationship between people and companion animals, and the intrinsic risk of disease transmission [6]. The scope of the studies described in this thesis focuses on the serological detection of virus infections, particularly coronaviruses and influenza A viruses, that are (reverse) zoonotic or have (reverse) zoonotic potential and can infect companion animals, in order to gain more insight into the biology and prevalence of those viruses, and explore the usage of serological detection methods in a more general perspective.

1 Coronavirus

Coronaviruses (CoVs) are enveloped, single-stranded RNA viruses of mammals and birds, causing respiratory and/or gastrointestinal disorders, and in some cases leading to severe diseases in both animals and humans [7,8]. They belong to the subfamily *Orthocoronavirina*e in the family *Coronaviridae* of the order of *Nidovirales*, which can be further divided into four genera, *alpha-, beta-, gamma-* and *deltacoronavirus*, 25 subgenera and 45 species [9,10].

CoVs were long considered as of veterinary interest only, but are now generally recognized as zoonotic threats of pandemic potential. The 21st century has witnessed three major events of CoV emergence [11–13]. In the year 2002 and 2003, an outbreak of the severe acute respiratory syndrome coronavirus (SARS-CoV) resulted in over 8,000 infections with an 10% fatality rate in humans [12]. A decade later, Middle East respiratory syndrome coronavirus (MERS-CoV) emerged [13] and continues to influence human health with 2519 confirmed cases and 866 deaths (until January 2020, http://www.emro.who.int/health-topics/mers-cov/mers-outbreaks.html). The zoonotic origin of these two viruses was confirmed with the identification of SARS-like CoV in bats, and phylogenetic studies of MERS-CoV and related viruses [14,15]. In 2019, a novel human coronavirus (HCoV), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), emerged in Wuhan, China, causing a severe pandemic of respiratory disease (coronavirus disease 2019 (COVID-19)) [11,16]. As of 1 November 2020, nearly 46 million confirmed cases and 1.2 million deaths have been confirmed worldwide, which occurred via human-to-human transmission and mostly affected elderly and immunocompromised

people [17]. Extensive measures as (partial) lockdown and social distancing have been executed to control and reduce the ongoing outbreak.

Fortunately, SARS-CoV was eliminated after several months of discovery, and MERS-CoV failed to become established in human population because of the lack of efficiency in human-to-human transmission [18]. However, while SARS-CoV and MERS-CoV failed, SARS-CoV-2 took the world by surprise and spread efficiently to all the major continents. It is possible that SARS-CoV-2 will become endemic, as with the four other zoonotic respiratory viruses that successfully breached the species barrier and established themselves as true human coronaviruses [19,20]. These viruses, alphacoronavirus NL63 and 229E, betacoronavirus OC43 and HKU1, are currently maintained in the global population through continuous circulation [18]. HCoV-229E and HCoV-OC43 were first isolated in the 1960s [21,22], while HCoV-NL63 and HCoV-HKU1 were identified rather recent [23,24], as a result of boosted interest in coronavirus research after the SARS outbreak. These observations restated the tendency of CoVs to cross host species barriers [18]. Research into these human pathogens and their putative ancestors in animals will provide insights into the mechanisms of coronavirus cross-species transmission.

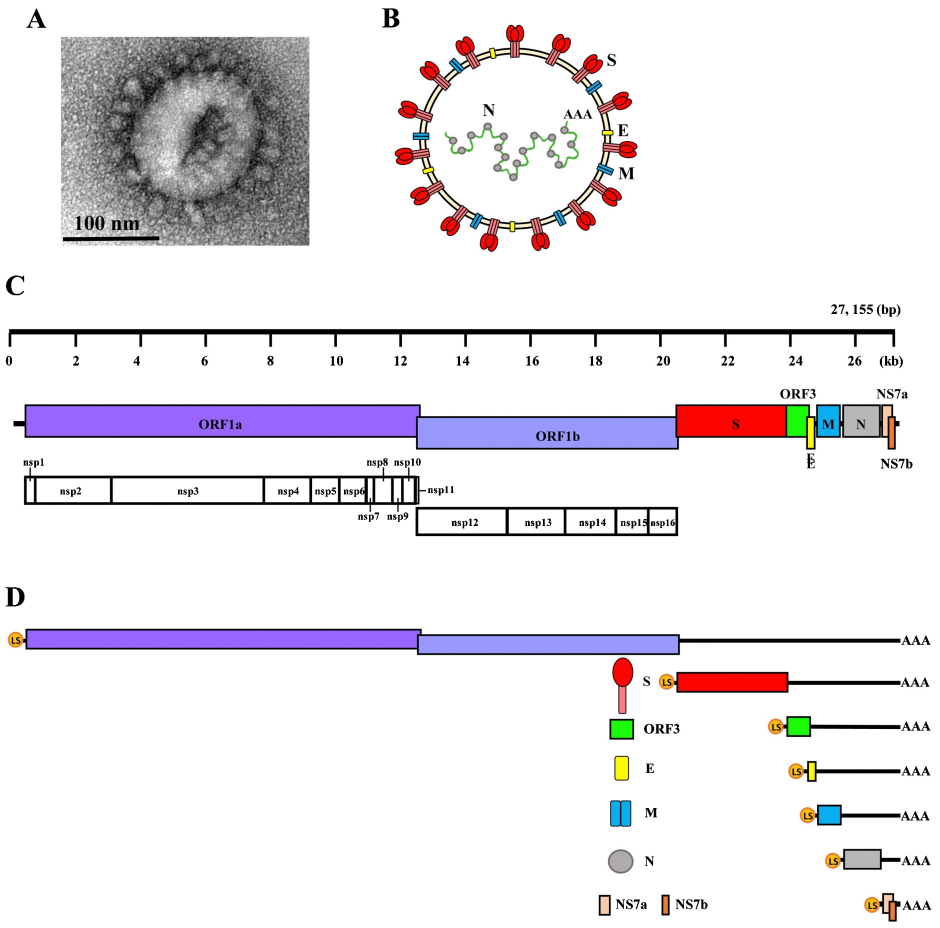

Fig 1. Schematic representation of CoV virion structure and genome organization. (A) Electron micrograph of a CoV virion (in this figure Swine enteric alphacoronavirus (SeACoV)). (B) Schematic depiction of CoV virion structure with different protein components. (C) Linear representation of the genome structure of SeACoV with the open reading frames (ORFs) annotated. (D) Genomic and subgenomic mRNAs with the first ORF(s), leader-body junction site (LS) and poly-A tail are shown corresponding to the genome structure. Figure was adopted from [25].

Coronavirus virions are spherical, enveloped particles of 120-160nm in diameter (Fig. 1 A, B)[26,27]. Contained in the envelope is a helical nucleocapsid that consists of the nucleocapsid protein (N), which packages the 5'-capped poly-adenylated positive-strand RNA genome into a compact core [28]. In addition, both the most abundant, triple-spanning membrane protein (M) and the small hydrophobic envelope protein (E) are required for virion assembly [27,29]. More relevant to this thesis is the surface protein located on the viral membrane, namely the spike (S) protein. Spike peplomers populate the virion surface, creating an image which resembles the shape of a royal crown or the solar corona, from which the name "coronavirus" is derived [26,29,30]. The expression of a fifth viral structural protein, hemagglutinin-esterase (HE), is unique to embecoviruses, a subset of betacoronaviruses [31].

The coronavirus viral genome is a unimolecular positive-strand RNA molecule of 26-32 kb in length [28]. Upon endocytic uptake, the viral envelope fuses with the limiting endosomal membrane and the genome is delivered into the cytoplasm where viral replication takes place. Viral proteins responsible for replication are encoded by the two largest open reading frames (ORF), ORF1a and ORF 1b (Fig. 1C) [27]. They are translated directly from the viral genomic RNA as mRNA into two precursor polyproteins, pp1a and pp1b [27]. Upon translation, both polyproteins are cleaved by viral proteases in order to produce functional replicase proteins [32]. The remaining virus genome is comprised of ORFs encoding structural and accessory proteins, which are translated from a set of nested, subgenomic mRNA's (Fig. 1D)[27,33,34]. This unique feature of viral replication allows design of recombination-based reversed genetics systems for generation of chimeric coronaviruses [35]. Coronavirus replication complexes are commonly associated with altered host cell membrane structures in the cytoplasm, where distinct spherical membrane invaginations or packed membrane vesicles can be found to support virus replication [36,37]. Virion assembly starts with packaging of newly generated full-length genomic RNA by the N protein, followed by budding of viral nucleocapsids at intracellular membranes [27]. At the end of the CoV infectious cycle, mature viruses are being released via the exocytosis pathway.

The CoV S proteins are heavily N-glycosylated homotrimeric class I fusion proteins, of which each monomer is between 1100 to 1600 amino acid residues in size [29]. S mediates entry into host cells through receptor binding and subsequent membrane fusion [38]. The S protein is the main target for CoV antibodies that can block infection [39], and as such the principle antigen for vaccine development. S protein is composed of two functional interdependent subunits: S1 and S2. During biosynthesis, S of

many CoVs is often cleaved at the S1/S2 boundary by cellular enzymes. The S1 subunit is responsible for receptor binding and recognition and thus determines the receptor binding specificity, while the S2 subunit mediates membrane fusion. At the start of CoV infection cycle, S protein first binds through its S1 subunit to a cell surface receptor, followed by fusion of the viral membranes with the host cell membrane through its S2 subunit [29].

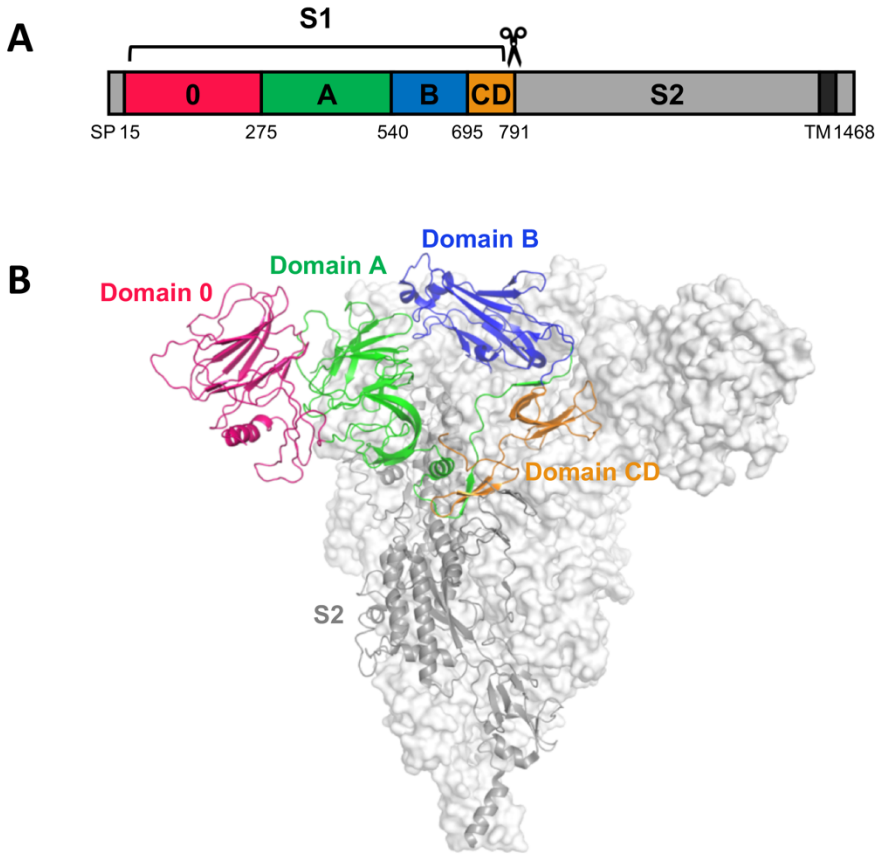

Fig.2. Schematic and structural representation of CoV spike. (A) Linear depiction of CoV S protein (FCoV strain UU2) with subunits S1 and S2, domains 0, A through D indicated. SP, signal peptide; TM, transmembrane domain; scissor, cleavage site. (B) Atomic model (surface and cartoon representation) of CoV spike (strain: FCoV UU4; PDB: 6JX7). Figures were generated using PyMOL. Different domains of the S1 subunit of one protomer are depicted in cartoon colored, with $S1^{0}$ shown in purple, $S1^{A}$ in green, $S1^{B}$ in blue, and the domains $S1^{CD}$ in orange. The S2 part of the protomer is marked in light gray.

Lately, with the single particle cryo-electron microscopy (Cryo-EM) technology, structures of S protein of many CoV representatives have become available [40–43]. Structural studies have confirmed that the S1 subunit of CoV S proteins can be divided into four domains designated A through D (Fig. 2), with the exception of S of most alphacoronaviruses that contain one or two N-terminal ($S1^{0}$) subdomains in addition [43]. Being the viral membrane distal domains, both domain A and B could function as receptor-binding domain (RBD) and contribute to S-receptor interaction, while $S1^{A}$ and $S1^{B}$

of the same S protein can bind to different attachment factors/receptors prior to virus entry [29,44–46]. In the meantime, it is reported that $S1^0$ of several CoVs is also involved in virus attachment [47,48].

Coronavirus entry into cells is mediated by their S protein which binds to receptors and triggers membrane fusion. Receptor engagement of the spike proteins has a major impact on their host tropism [29]. CoV often use proteinaceous receptors, with most of them being ectopeptidases. For example, both HCoV-NL63 (genus *alphacoronavirus*) and SARS-CoV (genus *betacoronavirus*) can utilize angiotensin-converting enzyme 2 (ACE2) as their entry receptor [49,50]. In addition, members of the *alphacoronavirus* and *deltacoronavirus* genera, like HCoV-229E and porcine deltacoronavirus (PDCoV), bind to aminopeptidase N (APN), whereas dipeptidyl peptidase-4 (DPP4) is used by the betacoronaviruses MERS-CoV and bat coronavirus HKU4 [51–54]. To date, not all the coronavirus receptors are so far revealed, such as the receptors of porcine epidemic diarrhea virus (PEDV) and serotype I feline coronavirus (FCoV) are still unknown. Besides binding to proteinaceous host molecules for cellular entry, many coronaviruses from different genera could also utilize sialylated glycans as their receptor/attachment factor [55].

Fig.3. Phylogenetic tree of animal CoVs. A phylogenetic tree of animal coronaviruses was constructed based on multiple protein sequence alignments of the ORF1b encoding polyprotein by the neighbor-joining method using MEGAX. Several HCoVs were included in the analysis as reference sequences. CoVs mostly included in this thesis were indicated side-by-side with animations of their hosts.

Noticeably, the CoV S-receptor interaction can be rather promiscuous, i.e. virus binding/entry can be mediated via receptor orthologs of different hosts. For instance, HCoV-229E and canine coronavirus (CCoV) can utilize feline APN for cellular entry, while the spike of PDCoV, a virus that emerged only in 2012, can bind to APN of different species like chicken and human [8,52,56]. In the meantime, CoVs from the genus *Embecovirus* utilize *O*-acetylated sialic acids (*O*-Ac-Sias) for infection [44,57]. It has been shown that *O*-Ac-Sias are abundantly present in mammalian cell lines and tissues, therefore functioning as viral receptors with different tissue tropism [58]. Such characteristics of CoVs supports their zoonotic potential as well as possible (reverse) zoonosis and led us to study CoV infections in companion animals.

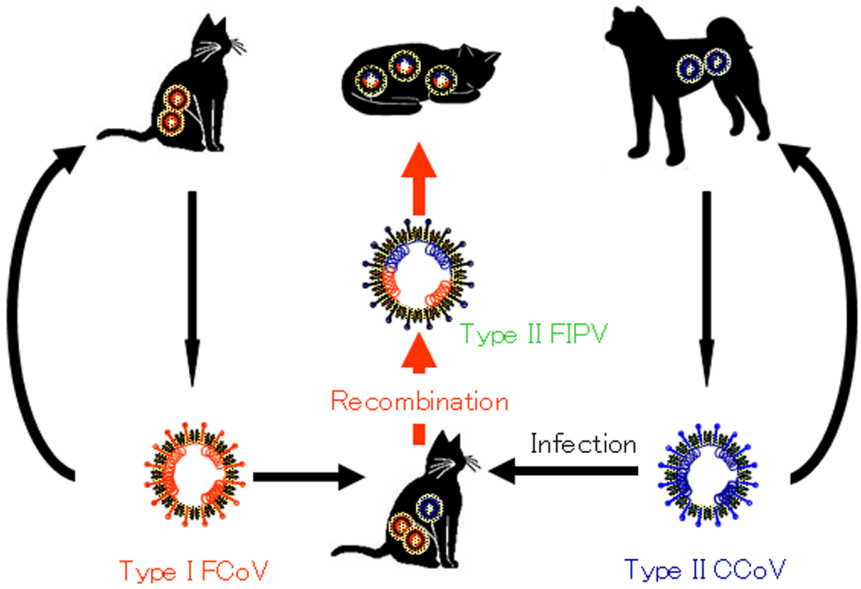

Fig. 4. Model of the emergence of serotype II FCoV. Cats persistently infected with serotype I FCoV can also be infected with serotype II CCoV from dogs. Within infected cats, serotype II FCoV emerges by homologous recombination and may cause FIP. Figure was adopted from [59].

2 Coronavirus in companion animals

2.1 CoVs in cats

Feline coronavirus (FCoV), the causative agent of endemic infections in felids, belongs to the *alphacoronavirus* genus of subfamily *Orthocoronavirinae* in the family *Coronaviridae*, together with a variety of other CoVs that can infect humans and other mammalian species (Fig. 3). Based on their pathogenicity, FCoVs are often divided into two biotypes, the feline enteric coronavirus (FECV), and the feline infectious peritonitis (FIPV) (Fig. 4). FECV is endemic in domestic cat populations around the world [60]. It transmits from one cat to other cats via the fecal-oral route and commonly infects intestinal enterocytes, in most cases resulting in either mild enteric disease or only subclinical infections [61,62]. Based on experimental studies, FECV virus shedding can be detected via RT-PCR in the feces

of infected cats from 2 days up to 2 weeks post-infection, followed by a decrease in viral loads and intermittent shedding for up to 20 weeks after this period [63,64]. In contrast with FECV, FIPV infection leads to systemic or even lethal diseases (feline infectious peritonitis, FIP) in cats. FIPV induces disseminated perivascular pyogranulomatous inflammation and exudative fibrinous serositis in the abdominal and thoracic cavities [60,65]. Cats commonly have a nearly 100% fatal rate once clinical signs start to develop. It has been shown that substitutions in open reading frame 3abc (resulting in the production of a truncated FECV 3c protein), as well as in the fusion peptide or the furin cleavage site of the spike protein, could cause the conversion from FECV to FIPV [66–68]. Mutations of 3abc and S protein coding genes often occur in combination, however it is also indicated that single mutations in either S or 3abc seems to be sufficient to have a drastic impact on FECV tropism. Those mutations also lead to improve FECV internalization and replication, which further assists systemic cell-to-cell spread of the virus [66].

Both FECV and FIPV occur in two different serotypes, namely type I and II (Fig. 4) [69,70]. Serotype I FCoVs contribute to the majority of infections (80–90% of naturally occurring clinical cases) worldwide, while serotype II FCoVs are relatively less prevalent and mainly occur in Asia [71,72]. It is commonly understood that serotype II FCoV strains arise from recombination events between FCoV serotype I with CCoV, following CCoV dog-to-cat cross-species transmission (Fig. 4) [59,70,73]. Serotype II FCoVs can be well sustained in cell culture, while for serotype I FCoVs only a number of highly tissue culture adapted strains can be propagated *in vitro* [74]. Serotype I and II FCoV infections can be distinguished by virus neutralization (VN) assays using type-specific feline sera or monoclonal antibodies against the S proteins [69,70]. In serological studies, the current method to differentiate infections is also by detection of neutralizing antibodies against this two FCoV types in samples [74]. So far, the biology and viral life cycle of Serotype II FCoV are better studied in comparison to serotype I.

As most CoVs, FCoV also has one surface projection: the S protein which mediates receptor binding (via the S1 subunit) and membrane fusion (via the S2 subunit) to allow viral entry into host cells. It has been identified that Serotype II FCoV utilizes feline APN as its target cell membrane receptor, which is broadly expressed at the brush border of small intestine [56]. The primary cell receptor for the more prevalent serotype I FCoV remains to be identified, although reports have proposed the Fc receptor CD16 (FcyRIII) as a potential functional receptor, and dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) as a possible co-receptor [75,76]. Although research of FCoV seems of less importance and sole veterinary interest nowadays, it served as a suitable CoV model system which facilitated fundamental understanding of CoV biology, i.e. the discovery of CoV S as a fusion protein [77].

Noticeably, FCoV is not the sole coronavirus that can infect cats. Previous studies have shown that both HCoV-229E and CCoV can infect cats after experimental inoculation, causing an asymptomatic infection [78,79]. It is also reported that these two viruses can employ feline APN for cellular entry [56]. Thus, cats might potentially become naturally infected with CoVs of other species which may lead to viral adaptation e.g., mutation or recombination, resulting in emergence of novel CoVs and potentially new diseases. In Chapter 2, we employed different CoV S proteins, the main target for (neutralizing) antibodies, in order to look at CoV infections in cats.

2.2 CoVs in dogs

Similar with the situation in cats, CoVs are also endemic in domestic dog populations worldwide. To date, two CoVs have been identified in dogs: CCoV which belongs to the *alphacoronavirus* genus and canine respiratory coronavirus (CRCoV) that belongs to the b*etacoronavirus* genus. CCoV was first reported in 1971, while the discovery of CRCoV was relatively recent in 2003 [80,81]. Both viruses are not generally recognized as lethal canine pathogens, however severe diseases associated with higher mortality rate can occur post infection, especially when dogs are co-infected with other viral or bacterial pathogens [82–84]. CCoV is commonly transmitted via the fecal-oral route and generally causes mild, self-limiting symptom like diarrhea, while CRCoV is transmitted by aerosol, leading to mild signs of upper respiratory disease like sneezing, coughing and nasal discharge [85–87]. Due to similarities in virus pathogenesis and early host immune response, CRCoV had also been recommended as a naturally occurring animal model of SARS-CoV-2 infection in humans [88,89]. In Chapter 4, we studied seroprevalence of CCoV in dog serum samples in the Netherlands.

Like FCoV, CCoV also appear in two serotypes, type I and II [90]. Serotype II CCoV employs APN as its cellular receptor, while the receptor for serotype I CCoV still remains to be discovered [56,91]. Reports have shown that a subgroup of serotype II CCoV is the result of genetic recombination of the S protein N-terminal domains between serotype II CCoV and transmissible gastroenteritis virus (TGEV), a pig CoV that replicates in small intestine and in lung cells, and also use APN as its receptor [73,92]. CRCoV does not have distinguished serotypes. Most of the strains isolated have high overall genetic similarity with each other, and also with bovine coronavirus (BCoV), its possible ancestor, and HCoV-OC43 [81,93,94]. As a member of subgenus *Embecovirus*, CRCoV expresses two surface glycoproteins on the viral membrane, S and HE.

2.3 CoVs in horses

Equine coronavirus (ECoV) was first isolated from a two-week-old diarrheic foal with enterocolitis in North Carolina, United States in 1999 [95]. Since then, sporadic outbreaks of ECoV in adult horses have been reported in the United States, Europe, and Japan [96–100]. ECoV transmits via the fecal-oral route, and clinical signs of ECoV infection includes anorexia, lethargy, fever and, less frequently,

diarrhea, colic and neurologic deficits, while the morbidity rate varies from 10% to 83% during outbreaks [101,102]. The general mortality rate is low and always associated with endotoxemia, septicemia or hyperammonemia-associated encephalopathy [103,104]. Though ECoV is well known to be associated with enteric infections, it could also be detected in a small proportion of horses with respiratory signs [101,105]. ECoV virus shedding can be detected in fecal samples or nasal swabs from sick horses as well as healthy horses, but with a strong association between clinical signs assumed to be related to ECoV infection and virus detection in fecal samples suggesting a possible etiological role of ECoV [101,105]. As with CRCoV, ECoV also belongs to subgenus *Embecovirus*, and possess both S and HE glycoproteins. ECoV could well be sustained in tissue culture, and experimental evidences show that its HE protein could bind to 9-*O*-Ac-Sias [58,95]. In Chapter 3, we studied ECoV infection via setting up an ELISA-based detection method based on ECoV S1 protein.

2.4 SARS-CoV-2 in companion animals

The new coronavirus SARS-CoV-2 has taken the world by storm via efficient human-to-human spread. SARS-CoV-2 is speculated to have an animal origin, but there is currently not enough evidence to precisely explain the route of SARS-CoV-2 transmission [16,106]. Besides humans, SARS-CoV-2 can also infect a variety of other animal species including companion animals like cats and dogs [107–111]. At the time of writing, many sporadic events of SARS-CoV-2 infection in animals have been reported [112]. In April and May 2020, farmed minks have been reported to be infected by SARS-CoV-2, apparently with a human origin, in two mink farms in the Netherlands [113]. Several months later, minks from up to 68 farms were tested SARS-CoV-2 positive (until October 2020). Noticeably, some workers at a mink farm are assumed to have acquired the virus from mink [114]. Such zoonotic and reverse zoonotic events of SARS-CoV-2 have shown that contact with SARS-CoV-2 infected mink is a risk factor for contracting COVID-19. In the meantime, cats and dogs were also shown susceptible to SARS-CoV-2: they can be infected under experimental conditions, while natural transmission of SARS-CoV-2 from humans to cats and dogs has been reported, resulting in asymptomatic infections in dogs, and both symptomatic and asymptomatic infections in cats [107–110,112]. Serology study In Italy showed 3.4% of dogs and 3.9% of cats had measurable SARS-CoV-2 neutralizing antibody [115]. A serological survey in cat in Wuhan also indicated that 11 out 102 samples had SARS-CoV-2 neutralizing antibodies [116]. Presently, there have not been reports on SARS-CoV-2 infection in horses.

Although cats and dogs are susceptible to SARS-CoV-2, there is currently no evidence that pets play a role in the spread of the virus. Nevertheless, the close contact between pets and owners and the interaction between pets from different households raises the general concern about the role of these animals in SARS-CoV-2 transmission, and stresses the need for long-term surveillance studies. In Chapter 4, we analysed the antibody prevalence of SARS-CoV-2 in cat and dog serum samples of different cohorts collected between April and May 2020 in the Netherlands.

2.5 Diagnosis of CoVs in companion animals

CoVs are endemic in companion animal populations. Currently, with the emergence of SARS-CoV-2, companion animals are also facing the threat of reverse zoonosis. Such situation demands for sensitive and specific CoV diagnostic methodologies. For FCoV diagnosis, the "golden standard" is still histopathology with immunohistochemical staining (IHC) for FCoV on affected tissue, which can only be performed post mortem [117]. Other diagnostic possibilities include the detection of virus genome and specific antibodies [118,119]. Measurements of antibodies in serum are useful tools for seroprevalence studies, and nowadays immunofluorescence and ELISA are commercially used in different diagnostic laboratories. So far, at least one commercial assay is available for FCoV molecular diagnosis; the real-time PCR method that is based on the M protein encoding genome [120]. Similar assays are also commercially available for the diagnosis of CRCoV, often as a comprehensive real-time PCR package for CRCoV detection, together with other pathogens that may cause canine respiratory diseases [121]. For ECoV, real-time PCR methods that allows efficient detection of ECoV in horse feces has been established [102]. However, for most of the CoVs, including SARS-CoV-2, viral nucleic acid is only detectable within a limited timeframe post infection, and serological assays are also required to shed light on the transmission rate of infection within animal populations. To date, the most specific serological assays are virus neutralization assays, which are also the gold standard in coronavirus serology, but also are time-consuming and often labour-intensive. A chimeric S2 protein-based ELISA assay has been developed for ECoV serology, and screening of equine serum samples in the US was performed [122].

Molecular diagnostics of SARS-CoV-2 in companion animals is a fast advancing field due to the increasing spread of SARS-CoV-2 in humans and the threat of zoonosis and reverse zoonosis potential. At the time of writing, diagnosis of SARS-CoV-2 infection is mainly conducted by molecular assays such as real-time PCR. Serological screening of SARS-CoV-2-specific antibodies in companion animals like cats and dogs is important to get insight into the prevalence of this infection and possible modes of transmission (human-to-animal, animal-to-animal and animal-to-human). To date, numerus serological assays based on different antigens such as the complete S ectodomain protein, the S1 protein, the S RBD protein and the N protein have been developed to study antibody levels pre-and post-experimental infection, and for screening of serum samples from possible naturally infected cats and dogs [116,123–128]. In chapter 4, we developed and validated our in-house method for SARS-CoV-2 serology, and analysed the SARS-CoV-2 seroprevalence in cat and dog serum samples.

3 Influenza A virus

Influenza A viruses (IAVs) are enveloped, negative-sense, single-stranded RNA viruses with a total genome size around 13.5 kb [129]. IAV is a member of family *Orthomyxoviridae*, which is featured by

a segmented RNA genome [130]. IAV contains eight RNA segments, which encode at least 10 viral proteins, including polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), polymerase acidic protein (PA), nonstructural protein (NS1), nucleoprotein (NP), matrix (M) and most importantly, the two surface proteins on the viral membrane, the hemagglutinin (HA) and the neuraminidase (NA) [129,131]. HA and NA engage in distinct functions in receptor binding and catalysis-driven virion elution, both of which are essential for optimal virus infection and fitness, and further determine virus tropism [132]. So far, 18 HA and 11 NA serotypes were identified from IAVs circulating in birds and mammals, and through reassortment many viral subtypes with different HA/NA combinations can emerge [133–137].

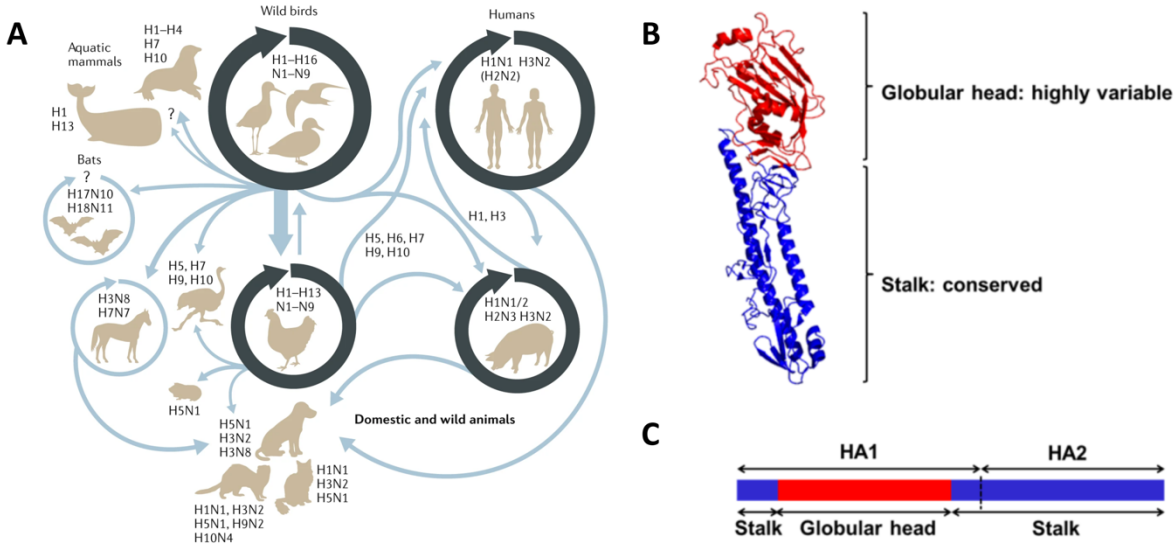

Fig.5. IAV ecology and HA structure. (A) Wild birds are natural reservoirs of all IAV subtypes, while different IAV subtypes are able to transmit into many different species, either via intermediate hosts or by requiring adaptive mutations (light blue arrows). Figure was adopted from [131]. (B) Each protomer of HA is comprised of two functional domains: the highly variable globular head domain (red) containing the RBS, and the conserved stalk domain (blue) is located in membrane proximal region. (C) Schematic representation of IAV HA protein. The globular head domain is entirely comprised by the HA1 subunit, while the stalk domain is formed by the remainder of the HA1 subunit and the HA2 subunit. Figure was adopted from [138].

IAVs are capable to cause seasonal epidemics, pandemics and sporadic zoonotic infections. Wild aquatic birds are their natural host reservoir, but they were shown able to infect many species including humans and other animals including companion animals (Fig. 5A) [131,139]. IAVs can easily cross the species barrier to infect new species, making it a likely dangerous virus for both mammals and birds. They often exhibit within-host genetic diversity because of their high mutation rates, efficient replication and big virus population sizes [129,131]. IAVs displays iconic antigenic drift phenomenon, as a result of its structural protein's ability to undergo rapid evolution due to the plasticity of the viral

replicase proteins. Previous studies have shown that changes in the structural proteins, involving reassortments (antigenic shift) and accumulating mutations, are the driving forces of previous pandemics [140]. Hence, surveillance of IAV is necessary in order to oversee the general IAV prevalence and possibilities of IAV adaptation to new environments and host species.

The infectious cycle starts by the HA protein binding to its receptor, the sialic acid moieties which are the terminal residues of glycan chains of glycoconjugates. Following attachment is fusion between the viral and endosomal membrane and the release of viral ribonucleoproteins into the cytoplasm [129]. As the main IAV receptor-binding surface protein, HA is the prime target of (neutralizing) antibodies [141–143]. HA is a homotrimeric protein of which each of the protomers is comprised by two subunits: HA1 and HA2 (Fig. 5C). HA1 subunit forms the immunodominant globular head domain, which is the least conserved IAV antigen and contains the HA receptor-binding site. The N- and C-terminal regions of HA1 and HA2 subunit form the HA stalk domain, which is more conserved and immune-subdominant (Fig. 5B and 5C) [141,143]. It had been shown that antibodies against the head domain of HA can be strongly neutralizing but are mostly subtype or even strain specific, whereas antibodies against the stalk domain often show cross-reactivity within and across HA subtypes [144]. In Chapter 5, we used the HA protein as antigen and studied the antibody prevalence of cat and dog serum samples against different IAV subtypes.

4 Influenza A virus in cats and dogs

Domestic cats and dogs are now generally recognized as IAV hosts. To date, many IAV subtypes have been reported to infect cats and dogs. A number of them can cause severe acute respiratory syndromes: H1N1 (cats and dogs), H3N2 (cats and dogs), H3N8 (dogs), H5N1 (cats and dogs) and H7N2 (cats) (reviewed in [145,146]). Molecular detection of human type pandemic H1N1 (H1N1pdm09) in domestic and stray cats are reported since 2009, most likely to be the consequence of reverse zoonosis [147–149]. An H1N1pdm09 outbreak was reported among caged stray cats, with a strong indication of cat-to-cat transmission [150]. Infection of cats with avian origin H3N2 and H5N1 viruses also occur, but their prevalence is not high [151–154]. In December 2016, an outbreak of the avian H7N2 subtype in a cat shelter was reported, where approximately 500 cats were infected, showing clinical signs like sneezing, coughing and running nose [155]. This outbreak led to the first confirmed case of IAV transmission from cat to human, where a veterinarian who treated an infected cat also became infected with the feline H7N2 virus [156]. Occasional spillover events of other IAV to cats were also reported, such as H5N6 and H9N2 subtypes [157–159]. Notably, none of the IAV subtypes are considered endemic in cats, which is different with the situation in dogs. IAV outbreaks in dogs have been reported for avian-derived H3N2 viruses as well as equine-derived H3N8 viruses, and both subtypes are now considered endemic in canine population [160,161]. Canine H3N2 viruses were shown able to infect

cats in the field, which indicates a possible intermediate role of cats as hosts to transmit this virus to cats and dogs [151]. Interestingly, both avian and equine H3 subtype IAVs had adaptive mutations in dogs [162–165]. It is likely that gradual accumulation of substitutions throughout the IAV viral genome may have resulted in specific adaptation to the canine population. In the meantime, dogs are also susceptible to H1N1pdm09, H5N1, H5N2 and H9N2, where the prevalence is not high and only several sporadic cases of natural infections were reported [159,166–168]. Besides all IAV subtypes mentioned above, previous studies have also shown that both cats and dogs can be naturally infected by reassortant IAVs, and in the future novel IAV subtypes that infect cats and dogs may arise as the result of genetic reassortment [169–172].

Apart from molecular diagnostics of cats and dogs with respiratory symptoms for potential IAV infection, implementation of large scale IAV serosurveillance in canine and feline populations could also aid in monitoring the overall threat of human exposure to emerging zoonotic IAVs. Commonly used serological approaches are ELISA assays, virus neutralization assays and, unique for IAV and a small group of viruses, the hemagglutination inhibition (HI) assay [173–175]. A commercial ELISA (ID screen influenza A antibody competition; IDvet, Grabels, France) is also available for screening of IAV antibodies in animals. To date, the majority of IAV infections in cats and dogs were found in Asia and North America, while in Europe only infections with H1N1pdm09, H3N8, and H5N1 have been reported [150,176–179]. However, the number of seroprevalence studies in Europe seem to be limited. In chapter 5, we expanded the toolbox of IAV serology in cats and dogs, and analyzed the seropositivity rates in European cat and dog samples.

5 Aims and outline of this thesis

Coronaviruses and influenza A viruses are notorious for crossing host species barriers and their potential emergence upon zoonotic introduction poses a substantial threat to public health. In addition to animal health implications, the close contact between companion animals and humans also has potential risks of zoonotic virus infection. High-quality serological assays are key to understand the prevalence of and immunity to virus infections. In this thesis, we focused on the development of robust and specific serological assays that can serve as a toolkit for rapid diagnosis of CoVs and IAVs infections. This will give more insight and knowledge on the occurrence of different CoVs and IAVs infections in companion animals and their association with clinical diseases. In **Chapter 2**, we set out to detect CoV infection in cats through profiling of antibody presence in cat serum samples. Recombinant CoV spike S1 proteins of different animal and human CoVs were used as antigens for screening of cat sera for the presence of antibodies against the respective proteins. Positive samples were further tested by virus neutralization assays. This investigation intends to extend our knowledge of CoV epidemiology, potential reservoirs, and cross-species transmission. **Chapter 3** describes the development and validation of an S1-protein-based ELISA method for the detection of specific antibodies against ECoV. With this method, we are able to provide a consolidated diagnostic test to confirm ECoV outbreaks, as a complement to qRT-PCR analysis of equine feces samples. In addition, this method will be used for future estimation of ECoV prevalence and incidence in various equine (sub) populations. In **Chapter 4**, we performed a survey to follow and study the spread of SARS-CoV-2 in companion animals. A set of serological assays including ELISA and virus neutralization were developed and validated and next used to conduct the first seroprevalence study in the Netherlands. This will provide information regarding the potential risk of animal infections for public health in the later stages of the pandemic, especially when SARS-CoV-2 transmission between humans is greatly reduced and virus reservoir in animals could become more important. Except for studies of CoVs, in **Chapter 5**, we also developed a pipeline of serological assays which allow broad to specific analysis of IAV-specific antibody responses in cats and dogs. In this pipeline, serum samples were tested first with HA- and HA1-specific ELISAs and subsequently analyzed by nanoparticle-based, virus-free HI assays. We also demonstrated the value of using comprehensive serological assays to analyze IAV antibodies. Finally, in **Chapter 6**, the main findings presented in this thesis are discussed and summarized, along with yet unpublished observations.

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Chapter 2

Serological Screening for Coronavirus Infections in Cats

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Abstract

Coronaviruses (CoVs) are widespread among mammals and birds and known for their potential for cross-species transmission. In cats, infections with feline coronaviruses (FCoVs) are common. Several non-feline coronaviruses have been reported to infect feline cells as well as cats after experimental infection, supported by their ability to engage the feline receptor ortholog for cell entry. However, whether cats might become naturally infected with CoVs of other species is unknown. We analyzed coronavirus infections in cats by serological monitoring. In total 137 cat serum samples and 25 FCoV type 1 or type 2-specific antisera were screened for the presence of antibodies against the S1 receptor binding subunit of the CoV spike protein, which is immunogenic and possesses low amino acid sequence identity among coronavirus species. Seventy-eight sera were positive for antibodies that recognized one or more coronavirus S1s whereas 1 serum exclusively reacted with human coronavirus 229E (HCoV-229E) and two sera exclusively reacted with porcine delta coronavirus (PDCoV). We observed antigenic cross-reactivity between S1s of type 1 and type 2 FCoVs, and between FCoV type 1 and porcine epidemic diarrhea virus (PEDV). Domain mapping of antibody epitopes indicated the presence of conserved epitope(s) particularly in the CD domains of S1. The cross-reactivity of FCoV type 1 and PEDV was also observed at the level of virus neutralization. To conclude, we provide the first evidence of antigenic cross-reactivity among S1 proteins of coronaviruses, which should be considered in the development of serological diagnoses. In addition, the potential role of cats in cross-species transmission of coronaviruses cannot be excluded.

1 Introduction

Coronaviruses (CoVs) are enveloped viruses with a positive-stranded RNA genome and classified into four genera (*alpha-, beta-, gamma- and deltacoronavirus*) within the subfamily *Orthocoronavirinae* in the family *Coronaviridae* of the order *Nidovirales*. CoVs are found in a variety of mammals and birds, in which they can cause respiratory, enteric and systemic infections [1-3]. Additionally, CoVs have proven ability for cross-species transmission, exemplified by the emergence of severe acute respiratory syndrome (SARS) coronavirus in 2002/2003, and of the Middle-East respiratory syndrome (MERS) coronavirus in 2012 [4]. Both viruses belong to the *Betacoronavirus* genus and have an animal origin. SARS coronavirus crossed over from bats via intermediate hosts to humans, became human-adapted and quickly spread worldwide before its containment. MERS coronavirus recurrently enters the human population via its dromedary camel reservoir host, with limited, non-sustained human-to-human transmission particularly in healthcare settings [5-7]. Apart from SARS- and MERS-CoV, all four globally endemic human CoVs (HCoV-OC43, HCoV-NL63, HCoV-229E and HCoV-HKU1) originate from animals [8-11]. In addition, cross-species transmission potential of CoVs is also illustrated by the occurrence of chimeric coronaviruses that resulted from recombination events between feline CoVs (FCoV) and canine CoVs (CCoV) [12,13].

In order to get insight into the frequency of interspecies transmission of coronaviruses within and between animal and human populations and the risk of subsequent development of a pandemic, it is useful to screen for coronavirus infections in animal species; especially those that are in close contact with humans. Serological assays that can detect virus-specific antibody responses against infection play an important role in these epidemiological studies [14].

Cats live in close contact with humans and often roam around freely in the environment. Hence cats are an interesting species to study for infections with coronaviruses. Infections with feline coronaviruses (FCoVs) are recognized and widespread [15,16]. FCoVs are classified into two types, type 1 and type 2, based on the genetic and antigenic difference of their spike (S) protein [17]. In the field, the majority of FCoV infections are caused by FCoV type 1, while FCoV type 2, derived from recombination events of type 1 FCoVs and CCoVs obtaining the S gene and some flanking regions of CCoVs, is less prevalent [18,19]. Depending on the virulence of the FCoV strain and the immune response of the cat, the clinical presentation can range from apparently asymptomatic, through diarrhea, to full-blown feline infectious peritonitis [20]. FCoVs are members of the genus *alphacoronavirus*, to which also HCoV-229E, porcine transmissible gastroenteritis virus (TGEV), and CCoV belong. The latter three viruses and FCoV type 2 have been proven to use feline aminopeptidase N (fAPN) as a functional receptor in vitro [21]. The receptor for type 1 FCoV has still not been identified [22]. Notably, previous studies have shown that HCoV-229E and CCoV could infect cats after experimental inoculation, causing an asymptomatic

infection [23,24]. Thus, cats might potentially become naturally infected with CoVs of other species which may lead to virus-host adaptation e.g., mutation or recombination, resulting in emergence of novel coronaviruses and potentially new diseases [19,25]. The extent to which infections with CoVs of other species occur in the field, has not been explored in previous epidemiological studies of CoV infections in cats [15,26–28].

Being the main envelope protein of coronaviruses, the spike (S) protein mediates cell attachment and membrane fusion to allow viral entry. S functions as the main determinant of cell-, organ- and host tropism. Additionally, it is also the major target of neutralizing antibodies. Spike comprises two functionally interdependent subunits, S1 and S2, with S1 responsible for receptor binding and S2 for membrane fusion [3,29]. The S1 subunit is the least conserved and the most variable immunogenic antigen between coronavirus species [30]. Therefore, the S1 subunit is well suited as an antigen to screen for coronavirus type specific antibodies [31].

In this study, CoVs infection in cats were detected through profiling antibody presence in serum samples from cats. Recombinant CoV spike S1 subunits of different animal and human CoVs were expressed in a mammalian expression system and used for screening of cat sera for the presence of antibodies against the respective proteins. Positive samples were also tested by virus neutralization assays to support the specificity of the reaction [32-34]. This investigation intends to extend our knowledge of CoV epidemiology, potential reservoirs, and cross-species transmission.

2 Materials and methods

2.1 Serum Samples

Specific FCoV type 1 and FCoV type 2 sera were obtained from specific pathogen free (SPF) cats previously infected with strain UU2 or RM and FIPV-1146 respectively [35,36]. In addition, for the serological survey, 137 feline sera were retrieved from the serum bank in our lab. These had all been collected from cats in the Netherlands. Most of the samples (> 80%) were from a study on antibody titer testing for feline panleukopenia virus. The other samples were send to our lab for FIP or FeLV-FIV diagnostics. Sera of uninfected SPF cats were included as negative controls. All samples were stored at -20°C until analysis.

2.2 Cells and Viruses

African green monkey kidney cells (Vero-CCL81), human hepatoma cells (Huh7), pig kidney epithelial cells (LLC-PK1), human embryonic kidney 293 cells stably expressing the SV40 large T antigen (HEK-293T) were maintained in Dulbecco modified Eagle medium (DMEM, Lonza BE12-741F) supplemented with 10% fetal bovine serum (FBS, Bodinco, Alkmaar, The Netherlands).

Virus strains used in this study have been described previously [37-39]. Briefly, recombinant porcine epidemic diarrhea virus (PEDV) (rPEDV-S^{DR13}-GFP) was propagated and titrated in Vero cells, and HCoV-229E in Huh7 cells. PDCoV was propagated and titrated in LLC-PK1 cells, but supplemented with 1μg/mL TPCK-treated trypsin (Sigma-Aldrich, Inc., St Louis, MO, USA) in DMEM.

2.3 Plasmids Constructs and Recombinant Protein Expression

Synthetic sequences of 12 coronavirus spike S1 subunits (HCoV-HKU1 (GB: YP_173238.1), MERS-CoV (GB:YP_009047204.1), SARS-CoV (GB: AAX16192.1), HCoV-OC43 (GB: AAR01015.1), HCoV-229E (GB: NP_073551.1), HCoV-NL63 (GB: YP_003767.1), TGEV (GB: ABG89325.1), PEDV (GB: AOG30832.1), BCoV (GB: P15777.1), PDCoV (GB: AML40825.1), FCoV type 1 (GB: FJ938060.1), FCoV type 2 (GB: AY994055.1)) and different domains of PEDV S1 subunit ($S1^0$ and $S1^{A-D}$, as identified and described in [40]) were cloned into pCAGGS expression plasmids as described previously [41]. Similarly, the expression constructs encoding chimeric proteins in which S1s were fused to the Fc domain of mouse IgG2a. For protein production, HEK-293T cells were transfected with plasmid DNA conjugated to polyethyleneimine (Polysciences, Inc., Warrington, PA, USA). At 8–16 h post transfection, inoculum was removed and the transfection mixture was replaced by 293 SFM II expression medium (Gibco®, Life Technologies Inc., Grand Island, NY, USA). At 6–7 days post transfection, cell supernatants were harvested and proteins were collected by Protein A Sepharose beads (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Proteins were then eluted with 0.1 M citric acid, pH 3.0 and neutralized with 1 M Tris-HCl, pH 8.8. Concentrations of proteins were assessed by Nanodrop spectrophotometry (ThermoFisher Scientific Inc., Waltham, MA, USA) and confirmed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with bovine serum albumin (BSA, BioIVT, West Sussex, UK) as standard. Typical yields for proteins were 0.2–0.5 mg/mL. For long term storage, proteins were stored at −80 °C upon usage.

2.4 FCoV S Structure Modelling and S1 Domain Expression

To study the potential cross-reaction between FCoV type 1 and 2 in more detail, models of FCoV type 1 (strain: UU2; GenBank accession no.: FJ938060.1) and FCoV type 2 (strain: 79-1146; GenBank accession no.: AY994055.1) S proteins were generated via the automated protein structure SWISS-MODEL Homology Modelling server (https:// swissmodel.expasy.org/) [42] using the elucidated HCoV-NL63 Cryo-EM structure (PDB code: 5SZS) as the input model. Figures were made with PymoL (The PyMOL Molecular Graphics System, Version 1.0 Schrödinger, LLC.). FCoV S1 domains of both type 1 and 2, namely $S1^{0-CD}$, were expressed as murine Fc fusion proteins in HEK-293T cells as described above.

2.5 Enzyme-Linked Immunosorbent Assay (ELISA)

High binding microtiter plates (Greiner Bio-one BV, Alphen aan den Rijn, The Netherlands) were coated overnight at 4 °C with equal molar amount of protein (0.25 pmol per well, diluted in phosphate buffered saline (PBS, pH 7.4)). After three washes with washing buffer (PBS containing 0.05% Tween-20), the plates were blocked for 2h at 37 °C with blocking buffer (PBS containing 5% milk powder (Protifar, Nutricia, Zoetermeer, The Netherlands), 0.05% Tween-20). Protein coating efficiency was assessed by binding of anti-mouse IgG antibodies in a direct ELISA, and confirmed the equimolar coatings of all proteins. To detect antigenic reaction with serum samples, sera were tested in duplicate at a 1:200 dilution in blocking buffer, and then incubated in the plates at 37 °C for 1h. After washing, plates were incubated with a 1:4000 diluted horseradish peroxidase (HRP)-conjugated goat anti cat IgG (Rockland Immunochemicals, Inc., Pottstown, PA, USA) at 37 °C for 1 h. The peroxidase reaction was then visualized via adding TMB Super Slow One Component HRP Microwell Substrate (BioFX®, Surmodics IVD, Inc., Eden Prairie, MN, USA) for 10 min. Reaction was stopped with 12.5% sulfuric acid and optical densities (OD) were measured at 450 nm. Negative sera (from uninfected SPF cats) were included to determine the ELISA cut-off values; sera with OD values higher than 5-fold the OD of negative sera were considered positive. All 12 S1 proteins were coated on the same ELISA plates making it easy to screen and compare the OD values of individual sera in one assay. Hereby we excluded the sera that give high background OD values against all proteins being considered false positive.

2.6 Virus Neutralization Assay

Neutralization assays were performed with some of the CoVs to support the specificity of ELISA results. Cat sera were serially diluted 2-fold in DMEM and mixed 1:1 with rPEDV-S^{DR13}-GFP, HCoV-229E or PDCoV (2000 50% tissue culture infective doses [$TCID_{50}$]/mL). These mixtures were then incubated at 37 °C for 1h, and 100 µL of each mixture was used for inoculation with Vero, Huh7 and LLC-PK1 cell monolayers in 96-well plates, respectively. For PDCoV infection, TPCK-treated trypsin (Sigma-Aldrich, Inc., St Louis, MO, USA) was supplied to LLC-PK1 tissue culture medium at a final concentration of 1µg/mL. At 2–5 days post infection, cytopathic effect (CPE) could be observed via microscopy. Virus neutralization titers (VNT) were expressed as the highest serum dilution resulting in 90% reduction of cytopathic effect (HCoV-229E and PDCoV) or virus-induced fluorescent cells (PEDV). Before virus neutralization, sera were inactivated through incubation at 56 °C for 30 min. Experiments were performed in triplicate.

3 Results

3.1 CoVs Seroprevalence in Cats

Feline sera (n = 137) were screened by indirect ELISA for antibody reactivity against 12 CoV S1 antigens. The OD values against these 12 antigens are shown in Figure 1. In total, 78 of the 137 sera (56.9%) contained anti-CoV antibodies, while 43 sera showed reactivity against more than one CoV S1

antigen. None of the samples had to be discarded because of reactivity against all of the proteins indicating a potential false positive result. The frequency of different combinations of CoV-S1 reactive samples is summarized in Table 1. Reactivity against eight out of 12 CoV S1 antigens could be observed, whereas none of the sera recognized the S1 protein of HCoV-HKU1, MERS-CoV, SARS-CoV and HCoV-OC43.

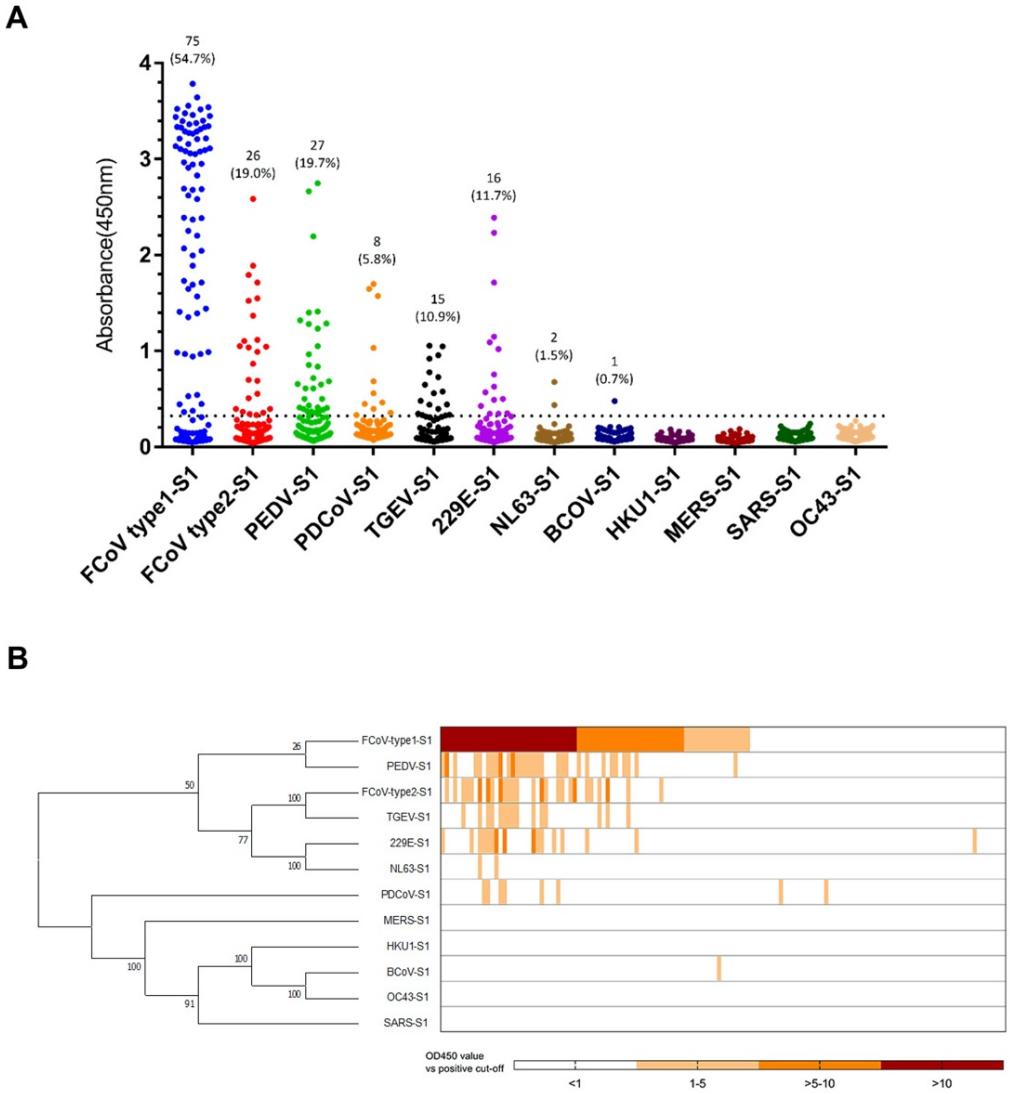

Figure 1. ELISA-reactivity of feline serum samples against S1 proteins of different coronaviruses. (A) Reactivity of feline serum samples (n = 137) against 12 coronavirus S1 antigens was determined by ELISA. The number and percentage of seropositive samples are indicated for each S1 protein. The dashed line is the positive cut-off. Each dot represents one individual sample within each antigen panel. (B) Relative ELISA (OD450 values divided by cut-off value) results are displayed as a heat map. Different S1 antigens were grouped by amino acid sequence phylogeny (left panel) using MEGA7. Each column of the heat map represents an individual sample, and columns were arranged in a descending order based on ELISA-reactivity against feline coronavirus (FCoV) type 1.

Table 1. Numbers of positive cat samples and different combinations of reactivity found. The number of positive sera against each individual S1 is shown in the bottom row. Positive ELISA reactions are colored in orange. Cut-off value was determine as the 5-fold over the OD450 of negative sera.

S1 antigens of different coronaviruses								Number of cats (total =137)
FCoV type1-S1	FCoV type2-S1	PEDV-S1	PDCoV-S1	TGEV-S1	229E-S1	NL63-S1	BCoV-S1	
								59
■								32
■	■							6
■		■						10
■	■	■						1
					■			1
			■					2
■		■			■			4
■	■	■		■	■			2
■	■		■	■	■			2
■	■	■		■	■	■		1
■			■		■			1
■	■				■			1
■	■	■			■			1
■		■			■	■		1
■	■	■	■	■	■			1
■					■			1
■	■	■	■	■				1
■	■	■	■	■	■			1
■							■	1
■	■	■		■				4
■	■			■	■			4
75	26	27	8	15	16	2	1	

As expected, many sera were positive for FCoV S1, with 75 sera (54.7%) positive for FCoV type 1 and 26 sera (19.0%) for FCoV type 2 S1 (Figure 1). All of the FCoV type 2 S1 positive sera also tested positive for FCoV type 1 S1, while 15 of 26 FCoV type 2 S1 positive sera also reacted with TGEV S1. The FCoV type 2 S1 and TGEV S1 ELISA reactivities showed a strong nonparametric Spearman correlation (Spearman r = 0.84, $P < 0.0001$). With respect to this, we suggest that the TGEV S1 positivity was due to cross-reactivity of FCoV type 2 S1, as FCoV type 2 shows close antigenic and genetic relationship with TGEV (S1 shares 70.4% amino acid sequence identity). The remaining 11 FCoV type 2 S1 positive but TGEV S1 negative sera do react with FCoV type 1 S1. An explanation might be the cross-reactivity between FCoV type 1 and type 2. Remarkably, 40 feline sera were reactive with S1 proteins from human, porcine and bovine CoVs (Table 1), including HCoV-229E (16/137), HCoV-NL63 (2/137), PEDV (27/137), PDCoV (8/137) and BCoV (1/137). OD values of feline sera positive for HCoV-NL63 S1 and BCoV S1 were relatively low (Figure 1A). ELISA reactivity towards non-feline CoV S1 proteins might be explained by infection with the respective or related CoVs or by the presence of cross-reacting antibodies, although there was low sequence identity (< 32.8%) between

S1 proteins of FCoV type 1 and related non-feline coronaviruses (for the complete comparison of S1 sequence identities, see Table 2). Yet, all of PEDV-S1 positive sera were also positive for FCoV type 1 S1 (Figure 1B, Table 1). The ELISA results of FCoV type 1 S1 and PEDV S1 showed a strong nonparametric Spearman correlation (Spearman r = 0.83, $p < 0.0001$). Thus, this might indicate the occurrence of antibody cross-reactivity against FCoV type 1 and PEDV S1 antigens. Many of the HCoV-229E and PDCoV S1 positive sera also reacted with FCoV type 1 S1, but no strong nonparametric Spearman correlation was observed (HCoV-229E, r = 0.216; PDCoV, r = 0.307). One feline serum only reacted with HCoV-229E S1, and two feline sera only recognized PDCoV S1. (Figure 1B, Table 1). This observation led us to hypothesize that cross-reactivity may not play a role in ELISA reactivity of these three sera, but that the three cats had been infected with these viruses or related viruses.

Table 2. Percentage identity matrix of every pair sequences of spike S1 subunit.

Sequence no.	Protein (accession no.)	% Amino acid sequence similarity with protein of sequence no.:										
		1	2	3	4	5	6	7	8	9	10	11
1	FCoV type1-S1 (FJ938060.1)											
2	FCoV type2-S1 (AY994055.1)	28.5										
3	PEDV-S1(AOG30832.1)	32.8	30.2									
4	PDCoV-S1 (AML40825.1)	23.1	26.7	26.5								
5	TGEV-S1 (ABG89325.1)	28.5	70.4	31.8	27.4							
6	HCoV-229E-S1 (NP_073551.1)	30.4	38.6	34.5	27.7	37.2						
7	HCoV-NL63-S1 (YP_003767.1)	28.4	30.4	29.6	26.7	31.7	51.9					
8	BCoV-S1 (P15777.1)	9.6	11.3	8.5	10.8	11.2	14	10.6				
9	HCoV-HKU1-S1 (YP_173238.1)	11	12.1	9.5	12.4	11.4	13.5	11.8	61.2			
10	MERS-S1 (YP_009047204.1)	9.2	9.2	7	6.6	9.4	7.9	8.1	15.6	17		
11	SARS-S1 (AAX16192.1)	6.5	8.9	9.1	8.6	8.9	11.4	8.5	17	17.9	15.5	
12	HCoV-OC43-S1 (AAR01015.1)	8.9	11.3	7.7	10.2	10.7	13.1	9.6	90.9	59.7	15.7	16.4

3.2 Assessment of Cross-reactivity for CoV S1s

In our screening, 43 samples were shown to be positive for two or more S1 proteins including FCoV type 1. The data prompted us to test different hypotheses which may explain this phenomenon: specific reaction through natural virus infection or reaction due to cross-reactivity with FCoV-S1 antigens. To explore this further, we employed 25 FCoV type 1 specific sera derived from specific pathogen free (SPF) cats that had been experimentally infected with FCoV type I strain RM ($n = 9$) or strain UU2 ($n = 16$). These sera were tested for their ELISA reactivity against seven CoV S1 proteins (excluding TGEV S1) that showed positive reactivity in the previous serological screening. As expected, all 25 sera were positive for FCoV type 1 S1 in our ELISA; interestingly, four samples also reacted with FCoV type 2 S1, and five samples with PEDV S1. No positive ELISA-reactivity was detected with S1 of

HCoV-229E, PDCoV, HCoV-NL63 or BCoV (Table S1). Thus, FCoV type 1 infection could lead to the generation of antibodies that cross-react in the S1-ELISA with FCoV type 2 and PEDV S1 proteins.

3.3 S1 Domain Mapping of Conserved Epitopes Shared between Type 1 and Type 2 Feline Coronaviruses

The ELISA cross-reactivity of FCoV type 1 specific sera with FCoV type 2 S1 antigens prompted us to map the domains responsible for cross-reaction within the S1 subunit. Hence, to identify domain borders within S1, we built homology-based models of both FCoV type 1 and type 2 spike using the related elucidated HCoV-NL63 cryo-EM structure as the template model. As shown in Figure 2A, continuous structural domains can be identified for the S1 subunit of both spikes, namely $S1^0$, and $S1^A$ through $S1^D$. Amino acid sequence identities of these domains between FCoV type 1 and type 2 differ, ranging from 22.4% to 57.4% (Figure 2B). Several S1 proteins for both type 1 and type 2 FCoV-S1 comprising one or two domains were expressed and purified (Figure 2C). FCoV type 1 specific sera (n = 4 for strain RM and n = 3 for strain UU2) and type 2 (n = 6, strain 79-1146) specific sera were then tested against these proteins in ELISA format. The four FCoV type 1 specific sera that cross-reacted with S1 of FCoV type 2 again showed binding to FCoV type 2 S1. But the FCoV type 2 specific sera showed little to no reactivity against FCoV type 1 S1. As shown in Figure 3, the type specific antisera reacted with all of the homologous S1 domains, with S1 and $S1^B$ of both type 1 and type 2 displaying the strongest reaction. Interestingly, the CD domain showed the highest level of cross-reactivity between FCoV type 1 and 2, in agreement with its highest sequence identity among S1 domains (Figure 3). The other three domains showed little to no cross-reactivity.

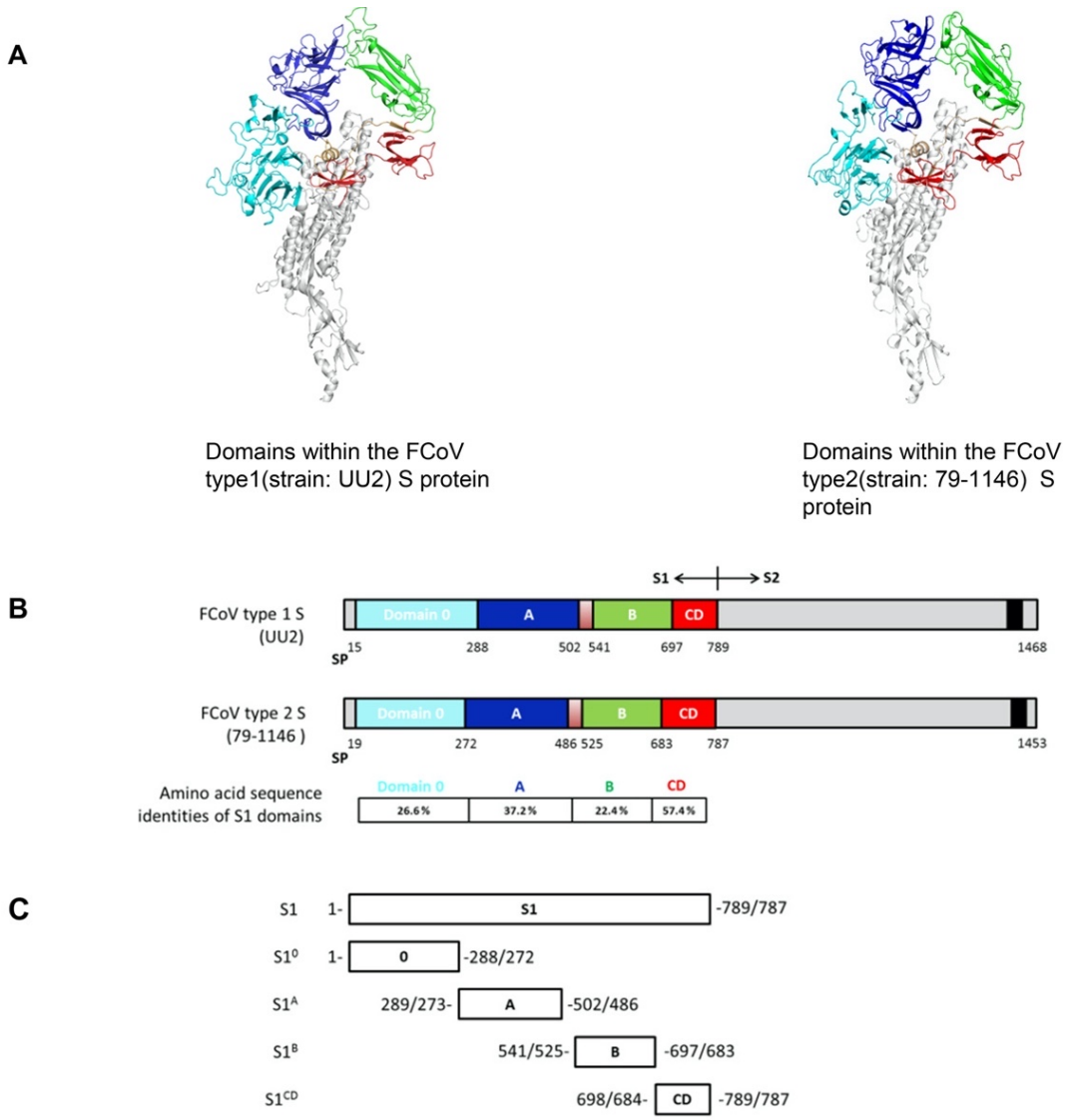

Figure 2. Domains within the FCoV type 1 and type 2 spike (S) proteins. (A) Structure model of two types of FCoV S trimer based on the HCoV-NL63 S structure were generated via the SWISS-MODEL Homology Modeling server using ProMod3. Figures were produced by PyMOL. Different domains of the S1 subunit of one protomer are colored, with $S1^0$ shown in cyan, $S1^A$ in blue, $S1^B$ in green, and the domains $S1^{CD}$ in red. The S2 part of the protomer is marked in light gray. (B) Schematic presentation of the FCoV type 1 (strain UU2) and type 2 (stain 79-1146) S protein with the signal peptide (SP), the S1 subunit (the domains are colored as described in the legend of Fig.2A) and the S2 subunit (the C-terminal transmembrane domain is indicated by a black box). Amino acid sequence identities between FCoV type 1 and type 2 S1 domains are indicated. (C) Diagram of the different S1 subdomains sequence. All S1 subdomains were C-terminally tagged with the Fc part of mouse IgG2a (not shown in the figure) and expressed as Fc fusion proteins.

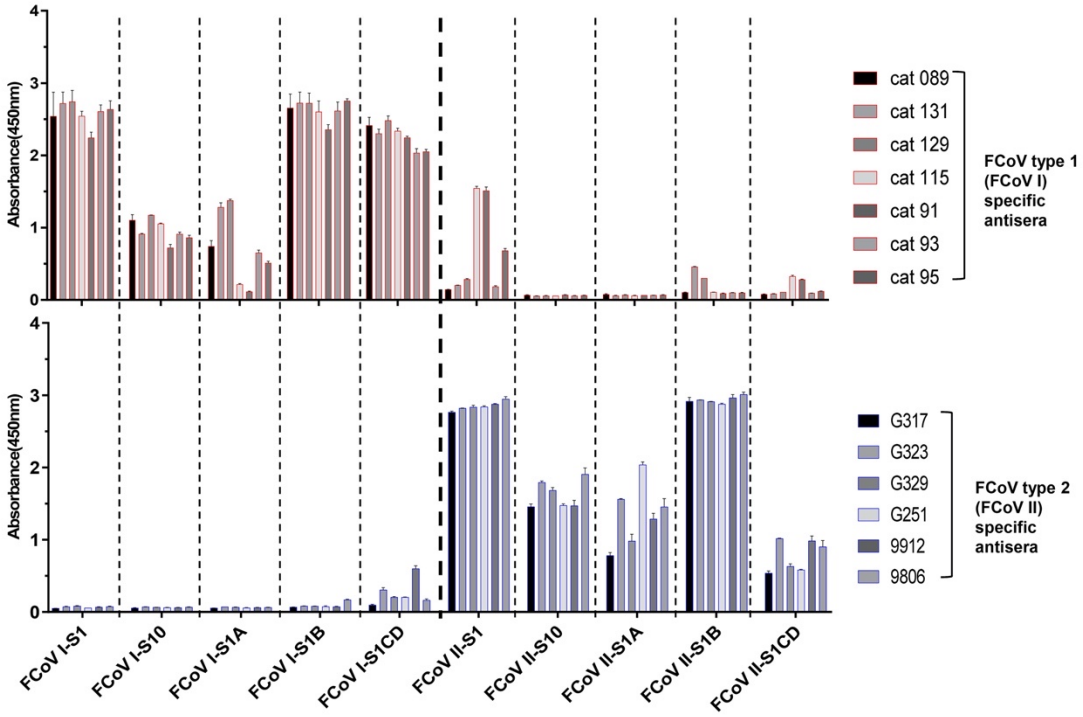

Figure 3. ELISA-reactivity of FCoV specific antisera against different S1 subdomains of FCoV type 1 and 2. Equimolar amount of purified S1 proteins and the four S1 subdomains were coated onto 96-well plates and antibody binding was determined by ELISA. The FCoV type 1 and 2 specific antisera used in the screening were derived from experimentally infected specific pathogen free (SPF) cats and are indicated at the right side of each panel, absorbance values and antigens in use are shown on the Y- and X-axis, respectively. Graphs represent the mean values from three independently performed experiments. Standard deviations are indicated as error bars.

3.4 Assessment of Cross-Reactivity between FCoV type 1 and PEDV

Because the FCoV type 1 specific cat sera also showed ELISA reactivity with PEDV-S1 (Table S1), we analysed the reaction of the five PEDV-S1 positive cats in more detail. Samples were analysed via ELISA using antigens comprising different PEDV-S1 domains, as described in our previous study [40]. Cat sera taken pre- and post- FCoV infection were collected and tested. As indicated in Figure 4, all five cats had developed PEDV-S1 reactivity to different extent after FCoV type 1 inoculation. Noticeably, all sera showed the highest OD values with the CD domain, while the other domains, including the $S1^B$ containing the presumed receptor binding domain (RBD)[40], were non-reactive (Figure 4). On the other hand, the swine PEDV positive control serum exhibits strong reactivity against all PEDV-S1 domains. The next question we asked was whether FCoV type 1 specific sera could neutralize PEDV infection in tissue culture, as they showed no reactivity with the $S1^B$ of PEDV spike. As shown in Figure 5, PEDV neutralizing antibodies were detected in three out of five FCoV type I specific cat sera.

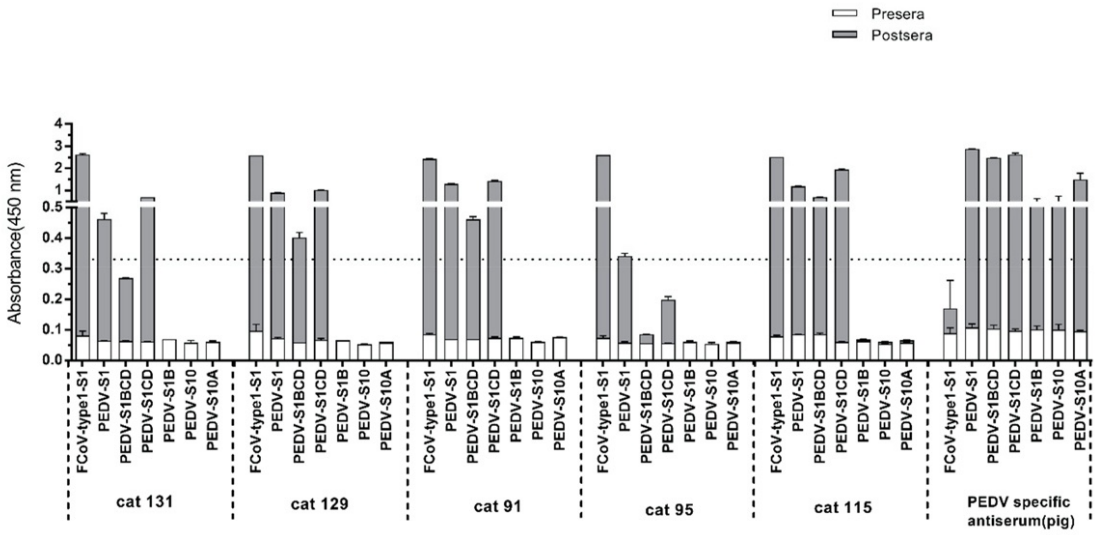

Figure 4. Reactivity of feline sera pre- and post FCoV type 1 infection to porcine epidemic diarrhea virus (PEDV) S1 domains determined by ELISA. Equal molar amount of purified S1 proteins and PEDV S1 subdomains were coated onto 96-well plates and antibody binding was tested by ELISA. Serum samples tested are indicated at the bottom of each panel. Moreover, absorbance at 450 nm and antigens are shown on the y- and x-axis, respectively. OD450 values of feline sera pre- and post FCoV infection were superimposed to one bar. Cat 91–131: sera from SPF cats pre- and post-experimental infection with FCoV type 1; PEDV specific antiserum: sera collected from a pig pre- and post-experimental inoculation with PEDV-S1. The dashed line shows the positive cut-off level. The graphs represent the means from three independent experiments. Error bars indicate standard deviations.

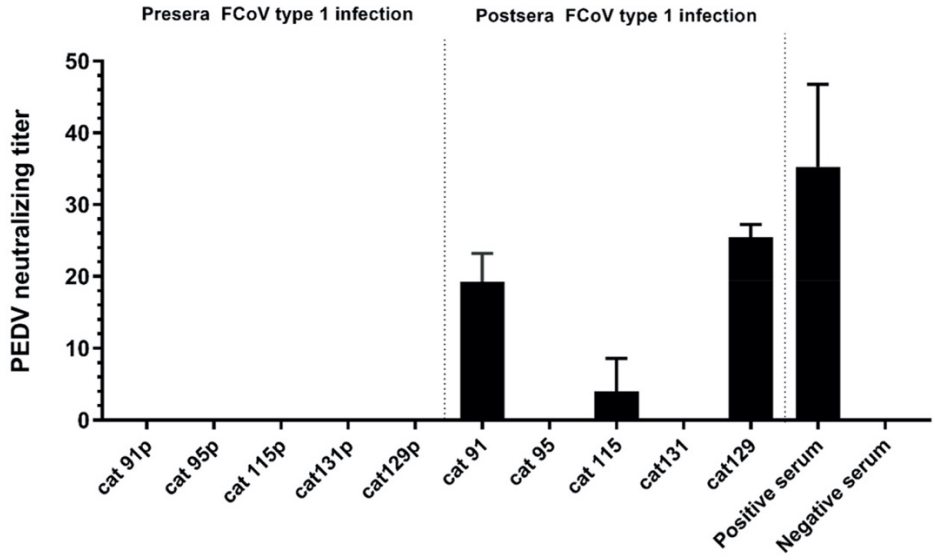

Figure 5. Neutralization of PEDV by cat antisera raised against FCoV type 1. rPEDV-S^{DR13}-GFP was mixed 1:1 with serial dilutions of serum prior to inoculation of Vero cells. Three days post infection,

cytopathic effect (CPE) could be observed and fluorescent cells were identified. The highest serum dilution inhibiting virus infections was recognized as the virus neutralization titer (VNT). The experiment was carried out in duplicate and repeated three times. Error bars indicate standard deviations. Sera were collected from SPF cats prior (cat 91–131p) and after (cat 91–131) experimentally inoculated with FCoV type 1. Positive serum: PEDV positive swine serum collected from the field; Negative serum: serum from FCoV negative SPF cat.

3.5 Evaluation of Virus Neutralization Capacity of HCoV-229E and PDCoV Positive Serum Samples

Several serum samples from field cats, but not virus-specific serum samples from FCoV inoculated SPF cats, were found to be ELISA positive for HCoV-229E (n = 16) and PDCoV S1 (n = 8) (Figure 1A). Also, a few feline sera displayed unique ELISA positivity for S1 of HCoV-229E (n = 1) or PDCoV (n = 2) (Figure 1B, Table 1). This could indicate that these antibodies were induced upon infection with these specific viruses. To corroborate the possibility of a natural infection in these cats with HCoV-229E or HCoV-229E-like viruses, we tested sera neutralization antibody titers. The results showed that one of the HCoV-229E S1 reactive feline sera was able to neutralize HCoV-229E infection (VNT = 32); no neutralization of PDCoV was detected for the all PDCoV-S1 positive sera.

4 Discussion

Coronavirus infections are endemic and ubiquitous in feline populations. Two viral types, type 1 and 2, are distinguished and both of them could well sustain themselves in the cat reservoir [15,43]. Both have been shown to have worldwide distribution, with the seropositivity rate up to 90% among animal shelter populations and in multi-cat households [20,44]. The majority of natural infections are caused by type 1 FCoVs, while in the field type 2 FCoVs are less common and mainly occur in Asia [15,28,45–47]. CoVs are generally considered to be host-specific; however, cross-species transmission does occur which may lead to incidental infections like the spillover of MERS-CoV from dromedary camel to humans, where humans function as an incidental and ultimately dead-end host [4]. But CoVs might also adapt to the new host exemplified by the animal origin of all four endemic human CoVs (HCoV-OC43, HCoV-NL63, HCoV-229E and HCoV-HKU1) [8–11]. Whereas in cats infections with FCoV are well recognized, studies regarding possible natural infections with other animal and human coronaviruses are lacking to the best of our knowledge. Knowing the genetic variability of coronaviruses and the use of orthologous receptors by non-feline CoVs, studies on cross-species transmission are desirable. This may provide insight regarding whether cross-species transmission does occur. In the present study we used the highly immunogenic S1 antigens to screen cat sera for the presence of antibodies against feline and non-feline coronaviruses, as a first indication of possible infections with these viruses.

In our study, 78 of the 137 cat sera were shown to be seropositive for coronaviruses. The seropositive rate (54.7%) against S1 of FCoV type 1 is consistent with previous studies [15,47]. All of the FCoV type 2 S1 positive sera of naturally infected cats were also positive for FCoV type 1 S1, which might be the result of cross reaction between the two proteins, despite their low amino acid identity. ELISA with specific antisera from experimentally FCoV type 1 and type 2 infected cats showed that sera of several FCoV type 1 infected cats could cross-react with FCoV type 2 S1. Domain mapping ELISA results showed that FCoV type 1 specific sera react to different levels with the S1 domains of FCoV type 1 S1 protein, and also reacts with FCoV type 2 $S1^{CD}$. Vice versa, FCoV type 2 specific sera also reacted with $S1^{CD}$ of FCoV type 1. These observations pose a potential two-way cross-reactivity between $S1^{CD}$ domains. Interestingly, in parallel with our findings on feline coronaviruses, we identified a number of samples that were seropositive against the S1 of PEDV, a viral pathogen that mainly replicates in the porcine intestinal epithelium. To study the possibility of cross-reaction, samples derived from pre- and post- FCoV infected cats were screened against PEDV S1 in ELISA. The reactivity found against PEDV S1 with FCoV specific sera shows that cross-reaction can occur at the level of domain $S1^{CD}$; the other PEDV S1 domains showed no reaction with the FCoV positive sera. Judging from these observations, it seems that $S1^{CD}$ plays an important role in cross-reaction between FCoV type 1 and 2, and also FCoV and PEDV. As $S1^{CD}$ is the most conserved domain among FCoV and also between FCoV and other alphacoronaviruses (for a systematic assessment of sequence identities, see Table 3), it is reasonable to hypothesize that antibodies can develop against conserved epitopes within this region and subsequently cause cross-reaction. This should be taken into account when developing and interpreting serological assays.

Table 3. Identities of amino acid sequences of FCoV type 1 (strain: UU2) S1 and S1 domains compared with the amino acid sequences of other alphacoronaviruses. (Identities are shown in %; NA: not available) The Genbank accession numbers of these viruses are as follows: FCoV type 1 (UU2), FJ938060.1; FCoV type 1 (RM), FJ938051.1; FCoV type 2, AY994055.1; TGEV, ABG89325.1; PEDV, AOG30832.1; HCoV-229E, NP_073551.1; HCoV-NL63, YP_003767.1.

	Amino acid % identity to FCoV type1(UU2)				
	S1	**$S1^0$**	**$S1^A$**	$S1^B$	$S1^{CD}$
FCoV type 1(RM)	89.3	88.3	85	94.9	91.2
FCoV type 2	28.5	26.6	37.2	22.4	57.4
TGEV	28.5	24	38.2	23.1	57.3
PEDV	32.8	20.4	36.5	24.8	51.2
HCoV-229E	30.4	NA	36.5	21.4	35.6
HCoV-NL63	28.4	19.3	37.7	20.6	44.9

Noticeably, ELISA reactivity among cat sera towards the N-terminal FCoV S1 domains 0 and A was less consistent and generally lower compared to whole S1, which seems to correlate with the higher antigenic variation in those domains found among FCoV type 1 strains [48] (Figure 3). Especially the sera from FCoV-RM infected cats (cat 91, 93, 95 and 115) showed lower OD values against $S1^A$. This

phenomenon could be explained as the samples displaying higher reactivity were from cats inoculated with FCoV-UU2 (cat 089, 131 and 129), the particular strain from which the S1 region was used as an antigen in the ELISA studies. In the meantime, the possibility of the variable ELISA reactivity might be due to the difference in individual antibody levels. In principle, the distinct antigenic reactivity of $S1^0$ and $S1^A$ between the two FCoV types might facilitate the development of a specific ELISA method which allows the serological discrimination of FCoV type 1 and type 2 infections in cats.

In order to provide further insight regarding cross-reactivity between FCoV type 1 and PEDV, we performed virus neutralization assays. Cross-neutralization of PEDV infection could be observed for some of the feline FCoV type 1 post-infection sera, in contrast to the pre-infection serum counterparts. Since FCoV specific PEDV neutralizing sera did not react with PEDV $S1^0$, $S1^A$ or $S1^B$, it is likely that the cross-neutralizing antibodies are targeting conserved epitopes in the $S1^{CD}$ domain or the S2 subunit of the PEDV spike protein [49]. Given the unknown TGEV infection background of the PEDV positive pigs, the cross-reaction of PEDV specific sera against FCoV type 1 could not be explored in our study, as TGEV positive pig samples would certainly influence the outcome [12,18,50]. Of note, our findings cannot exclude the possibility that field cats might incidentally get naturally infected with PEDV or PEDV-like viruses, as there had been one report showing the detection of PEDV in one stray cat via PCR assay [51]. It would be interesting to include more sera of cats from pig farms in future studies.

Considering the fact that cats play an important role in human society and have constant interaction with humans, it is of interest to conduct serological surveys for possible reverse zoonosis of human pathogens. In our study S1 antigens of several human coronaviruses were included and this led us to identify HCoV-229E seropositive feline samples in our ELISA survey (Table 1); one serum in particular reacted solely with HCoV-229E S1 but not with any other coronavirus. Of the HCoV-229E S1 reactive feline sera one showed low neutralizing activity against HCoV-229E infection. This might suggest that positive cats were indeed exposed to HCoV-229E or related viruses. MERS seropositivity is also seen in other species besides the dromedary host [52]. Rare cases of seropositivity might be considered as spill-over infections from the dromedary camel reservoir. Similar (perhaps dead-end) spill-over infections of 229E from the human reservoir to cats might also occur. A similar principle could also apply for PDCoV, a porcine pathogen that emerged rather recently. Both HCoV-229E and PDCoV use APN as their receptor and have been reported to also be able to use feline APN for cellular entry [21,37]. Although reports are lacking regarding the natural infection of these two viruses in cats, HCoV-229E was shown to cause a priming effect of FCoV antibody in experimentally FCoV infected cats suggesting that infection occurred [24]. Therefore, the detection of antibodies against S1 of HCoV-229E in a portion of the cats might be specific and due to the exposure to HCoV-229E through daily interaction with humans. Eight cats were seropositive for PDCoV of which two cats were seropositive only for PDCoV and not for any other CoVs. This could be caused by infection with PDCoV or PDCoV-related

viruses through avian sources, considering the fact that cats are natural avian predators and the presumed avian origin of PDCoV [2,37]. Our findings emphasize the potential role of cats as incidental hosts for non-feline coronaviruses and the need of in-depth study of naturally infected pathogens in cats. Besides serological studies efforts should also focus on isolation and identification of these viruses in cats.

In conclusion, we presented a thorough serological survey in cats using S1 proteins of different animal and human coronaviruses. We demonstrated, despite the low amino acid identity, cross-reactivity between S1 proteins of FCoV type 1 and 2, and between that of FCoV type 1 and PEDV. This should be considered when developing FCoV serological assays as well as interpreting the results. Our observation that some feline sera displayed antibody reactivity exclusively against non-feline CoV S1 proteins warrant further research into the epidemiology and cross-species transmission of coronaviruses in cats and other animals that are in close contact with humans. Further large scale serological studies regarding coronaviruses infection across animal species using arrays of CoV S1 antigens can shed light into the hitherto unresolved host promiscuity of coronaviruses and the risk of cross-species transmission.

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Supplementary information

Table S1. ELISA reactivity (OD450 values) of 25 FCoV type 1 specific antisera against S1 antigens of different coronaviruses. Table represent the mean OD450 values from three independent experiments. Positive ELISA reactions are colored in orange. Cut-off value is 0.36 and was determine as the 5-fold over the OD of negative sera. The Genbank accession numbers of these viruses are as in table 3.

serum samples	FCoV type 1 strains for infection	S1 antigens of different coronaviruses						
		FCoV type1 (UU2)-S1	FCoV type2-S1	PEDV-S1	HCoV-229E-S1	PDCoV-S1	HCoV-NL63-S1	BCoV-S1
cat 91	RM	2.603	1.995	1.906	0.100	0.073	0.148	0.100
cat 93	RM	2.923	0.231	0.198	0.116	0.075	0.096	0.120
cat 95	RM	2.931	0.732	0.440	0.116	0.062	0.059	0.053
cat 115	RM	2.619	1.786	1.817	0.128	0.116	0.084	0.114
Cat 039	RM	1.221	0.173	0.153	0.053	0.098	0.099	0.093
Cat 055	RM	1.220	0.129	0.077	0.085	0.091	0.114	0.065
Cat 057	RM	1.001	0.173	0.099	0.066	0.072	0.093	0.092
Cat 073	RM	1.905	0.240	0.123	0.096	0.109	0.113	0.064
Cat 109	RM	1.650	0.189	0.116	0.068	0.060	0.082	0.092
cat 129	UU2	2.537	0.401	0.976	0.143	0.053	0.062	0.050
cat131	UU2	2.858	0.262	0.880	0.070	0.080	0.150	0.080
cat 89	UU2	2.246	0.125	0.244	0.071	0.106	0.087	0.130
cat 024	UU2	1.812	0.090	0.156	0.107	0.114	0.100	0.127
cat 058	UU2	1.607	0.110	0.139	0.074	0.069	0.071	0.115
cat 066	UU2	1.292	0.065	0.066	0.101	0.144	0.080	0.085
cat 070	UU2	1.924	0.102	0.090	0.091	0.086	0.122	0.050
cat 164	UU2	1.151	0.095	0.074	0.085	0.078	0.099	0.051
cat 178	UU2	1.371	0.127	0.147	0.114	0.082	0.081	0.068
cat 188	UU2	1.277	0.095	0.078	0.129	0.056	0.056	0.068
cat 200	UU2	0.640	0.096	0.073	0.142	0.126	0.096	0.060
Cat 1	UU2	2.091	0.263	0.144	0.120	0.149	0.091	0.077
Cat 2	UU2	1.846	0.172	0.155	0.090	0.083	0.100	0.144
Cat 3	UU2	1.566	0.117	0.082	0.058	0.074	0.051	0.075
Cat 4	UU2	2.284	0.099	0.157	0.054	0.094	0.134	0.063
Cat 5	UU2	2.319	0.105	0.099	0.095	0.067	0.116	0.058

Chapter 3

Development and Validation of a S1 Protein-Based ELISA for the Specific Detection of Antibodies against Equine Coronavirus

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Abstract

Equine coronavirus (ECoV) is considered to be involved in enteric diseases in foals. Recently, several outbreaks of ECoV infection have also been reported in adult horses from the USA, France and Japan. Epidemiological studies of ECoV infection are still limited, and the seroprevalence of ECoV infection in Europe is unknown. In this study, an indirect enzyme-linked immunosorbent assay (ELISA) method utilizing ECoV spike S1 protein was developed in two formats, and further validated by analyzing 27 paired serum samples (acute and convalescent sera) from horses involved in an ECoV outbreak and 1084 sera of horses with unknown ECoV exposure. Both formats showed high diagnostic accuracy compared to virus neutralization (VN) assay. Receiver-operating characteristic (ROC) analyses were performed to determine the best cut-off values for both ELISA formats, assuming a test specificity of 99%. Employing the developed ELISA method, we detected seroconversion in 70.4% of horses from an ECoV outbreak. Among the 1084 horse sera, seropositivity varied from 25.9% (young horses) to 82.8% (adult horses) in Dutch horse populations. Further, sera of Icelandic horses were included in this study and a significant number of sera (62%) were found to be positive. Overall, the results demonstrated that the ECoV S1-based ELISA has reliable diagnostic performance compared to the VN assay and is a useful assay to support seroconversion in horses involved with ECoV outbreaks and to estimate ECoV seroprevalence in populations of horses.

1 Introduction

Coronaviruses (CoVs) are enveloped, positive single-stranded RNA viruses that belong to the subfamily *Orthocoronavirinae* in the family *Coronaviridae* of the order Nidovirales. They are classified into four genera (*alpha-, beta-, gamma- and deltacoronavirus*) and infect both mammalian and avian hosts [1,2]. Equine coronavirus (ECoV) belongs to *Betacoronavirus 1* species, within the *Embecovirus* subgenus of the *Betacoronavirus* genus, as does human coronavirus OC43, HKU1 and bovine coronavirus [3]. ECoV was isolated for the first time from a two-week-old diarrheic foal in North Carolina (USA) in 1999, suggesting the role of ECoV in causing enteric disease [4]. Since 2010, several cases of ECoV infections have also been reported in adult horses from the United States, Europe and Japan [5–9]. Equine coronavirus has been detected in fecal samples from horses with clinical signs that included anorexia, lethargy, fever and, less frequently, diarrhea, colic and neurologic deficits [10,11]. The morbidity rate varies from 10% to 83% during outbreaks. Mortality is low and has been related to endotoxemia, septicemia or hyperammonemia-associated encephalopathy [12,13]. The outbreaks in adult horses demand further studies on the pathogenesis and epidemiology of ECoV infections. For this, diagnostic assays with high sensitivity and specificity are crucial.

ECoV is known to be associated with enteric infections but can also be detected in a small percentage of horses with respiratory signs. Virus shedding can be observed in fecal samples or nasal swabs from sick horses as well as healthy horses, but with a strong association between clinical signs assumed to be related to ECoV infection and virus detection in fecal samples suggesting a possible etiological role of ECoV [10,14]. Recently, real-time quantitative PCR (qPCR) methods have been established and were shown to be able to detect ECoV in feces efficiently. However, ECoV viral nucleic acid is generally only detectable by qPCR within a limited timeframe of 3–9 days post infection, as reported from both field and experimental studies [6,7,12,15]. On the other hand, serological assays can be used to support the diagnosis of a clinical ECoV infection by showing seroconversion or a significant increase in antibody titer in paired serum samples. Serological assays are also needed to gain more insight into the transmission rate of infection within animal populations [16]. Antibodies induced by betacoronaviruses persist in blood for a longer period after infection [17,18]. The virus neutralization (VN) assay has long been used as a gold standard to confirm serological responses to coronavirus infections [19–21]. Although the VN assay is highly specific for the detection of antibodies, it is also time-consuming and laborious to perform. Alternative high-throughput serologic assays that correlate well with neutralizing antibodies are therefore needed. Severe infections of ECoV have been shown to be associated with high viral load, but mild or asymptomatic infections may occur with low levels of virus replication being negative in PCR and with variable immune responses [12]. Consequently, specific, sensitive and high-throughput serodiagnostic methods are necessary to avoid the underestimation of prevalence in surveillance studies.

The spike protein (S) of coronaviruses is the key mediator in virus cell entry and therefore the major target for neutralizing antibodies. The S ectodomain consists of two functionally interdependent subunits, S1 and S2. The N-terminal S1 subunit is responsible for receptor binding, while the C-terminal S2 subunit mediates membrane fusion [22,23]. The S1 subunit is the most variable immunogenic antigen among coronaviruses, and therefore it is an ideal candidate for the detection of CoV species-specific antibodies [24,25]. The objective of the study was to develop and validate an ELISA method for the detection of specific antibodies to ECoV and provide a tool for the diagnosis and the future estimation of ECoV prevalence and incidence in various equine (sub) populations.

2 Materials and Methods

2.1. Equine Serum Panels

A total of 1138 equine serum samples were included in this study. The details of serum panels A–H (*n* = 1084) are shown in Table 1. They were retrieved from the serum bank at GD Animal health Deventer, the Netherlands. All of them were collected for the monitoring of other diseases independent to this study, and their ECoV exposure status was unknown. With the exception of panel H (collected from Iceland), all serum samples from panel A to G were collected from horses in the Netherlands. Additionally, panel I included 27 paired (acute- and convalescent-phase) serum samples that were collected during an ECoV outbreak in the USA (2014). All samples were stored at −20 °C until tested.

2.2. Cells and Virus

ECoV strain NC99 was propagated and titrated in human rectal adenocarcinoma (HRT-18G) cells. HRT-18G cells and human embryonic kidney 293 cells stably expressing the SV40 large T antigen (HEK-293T) were maintained in Dulbecco modified Eagle medium (DMEM, Lonza, Basel, Switzerland) containing glutamine and supplemented with 10% fetal bovine serum (FBS, Bodinco, Alkmaar, The Netherlands), penicillin (100 IU/mL), and streptomycin (100 µg/mL).

The ECoV NC99 and HRT-18G were obtained from Dr. Udeni B.R. Balasuriya, School of Veterinary Medicine, Louisiana State University, USA [3,4].

2.3. Plasmids Design and Protein Expression

The sequence of the S1 subunit of the spike protein of the ECoV NC99 strain (residue 1–762 of the amino acid sequence) was derived from Genbank (Genbank No.: EF446615.1). Human codon-optimized sequences encoding the ECoV S1 subunit were synthesized and fused to the Fc domain of mouse IgG2a, which was subsequently cloned into the pCAGGS mammalian expression vector as described before [26]. For ECoV S1-Fc protein production, expression plasmid was transfected into HEK-293T cells using polyethyleneimine (Polysciences, Inc., Warrington, PA, USA) in a ratio of 1:10. After 6 h of incubation, the transfection medium was removed and replaced by 293 SFM II expression

medium (Gibco®, Life Technologies Inc., Grand Island, NY, USA). At six days post transfection, cell culture supernatants were harvested and the soluble S1 was purified from the culture medium using Protein A Sepharose beads (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Subsequently, the proteins were eluted using 0.1M citric acid, pH 3.0, and immediately neutralized with 1 M Tris-HCl, pH 8.8. The purity and integrity of proteins were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with GelCodeBlue stain reagent (ThermoFisher Scientific Inc., Waltham, MA, USA). Purified proteins were quantified by Nanodrop spectrophotometry (ThermoFisher Scientific Inc., Waltham, MA, USA) and by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with bovine serum albumin (BSA) as standard, then stored at −80 °C until further usage.

2.4. Virus Neutralization (VN) Assay

Equine sera (n = 231) were randomly selected from different serum panels (A–D) and tested for neutralizing antibody titers in an ECoV VN assay. Heat-inactivated equine sera (56 °C for 30 min) were serially diluted 2-fold in DMEM supplemented with 2% fetal bovine serum and mixed with an equal volume of ECoV NC99 strain (100 50% tissue culture infective doses ($TCID_{50}$)/well) in 96-well cell culture plates (Corning Inc., Kennebunk, ME, USA). Virus–serum mixtures were incubated at 37 °C for 60 min. Then 100 µL of the virus–serum mixture was added in duplicate to HRT-18G cells monolayers in 96-well cell culture plates. At six days post infection, a clear cytopathic effect (CPE) was observed and the virus neutralization titers (VNT) were determined. The VNT of sera were expressed as the reciprocals of the highest serum dilution that resulted in 90% neutralization of CPE. A titer of ≥8 was considered to be positive.

2.5. ECoV S1 ELISA Development

Two different formats were developed employing ECoV S1 protein, a so-called wet format ELISA (wELISA) and a dry format ELISA (dELISA).

2.5.1. ECoV S1 Wet Format ELISA (wELISA)

High-binding microtiter plates (Greiner Bio-one BV, Alphen aan den Rijn, The Netherlands) were coated with ECoV S1 protein (100 µL per well) in phosphate buffered saline (PBS, pH 7.4) overnight at 4 °C. The optimal protein amount and dilution of secondary antibody conjugate were determined by checkerboard titration. The protein concentration in use was 0.25 µg/mL. After three washes with PBS containing 0.05% Tween-20 (PBST), the plates were blocked with PBST containing 5% milk powder (Protifar, Nutricia, Zoetermeer, The Netherlands) for 2 h at 37 °C. Following blocking, plates were incubated with serum samples diluted 1:200 in PBST containing 5% milk powder for 1 h at 37 °C. After a washing step, 100 µL/well 1:20,000 diluted horseradish peroxidase (HRP)-conjugated goat anti-horse IgG (H&L) (Abnova, Taiwan, China) was added to detect bound antibodies and plates were incubated

for 1 h at 37 °C. Subsequently, the plates were washed, and the peroxidase reaction was then visualized via incubating plated with TMB Super Slow One Component HRP Microwell Substrate (BioFX®, Surmodics IVD, Inc., Eden Prairie, MN, USA) for 10 min at room temperature. The reaction was stopped by adding 12.5% sulfuric acid (H_2SO_4 (VWR International BV, Amsterdam, The Netherlands)) and optical densities (OD) were immediately measured at 450 nm using an ELISA microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). All serum samples were tested in duplicate.

2.5.2. ECoV S1 Dry Format ELISA (dELISA)

High-binding microtiter plates (Greiner Bio-one BV, Alphen aan den Rijn, The Netherlands) were coated with ECoV S1 protein (100 µL per well) in ammoniumcarbonate solution (9.8 g/L (VWR International BV, Amsterdam, The Netherlands)) overnight at 4 °C. Then 100 µL/well blocking solution (9.8 g/L ammoniumcarbonate + 4 g/L caseine (VWR International BV, Amsterdam, The Netherlands) + 20 g/L sucrose (Merck and Co., Inc., Kenilworth, NJ, USA)) was added and plates were incubated for one hour at room temperature. Subsequently, the contents of the plates were discarded, and plates were dried for four hours at 37 °C, vacuum sealed and stored at 4–8 °C. The optimal protein amount and dilution of secondary antibody conjugate were determined by checkerboard titration. The protein concentration in use was 0.13 µg/mL. Plates were incubated with serum samples 100 µL per well and diluted 1:200 in PBS + 0.05% Tween-20 + 2.5% dry milk (Bio-Rad Laboratories, Inc., Hercules, CA, USA) for 1 h at 37 °C. After a washing step (five times with PBST 300 µL/well on a Biotek automatic washing station), 100 µL per well 1:60,000 diluted horseradish peroxidase (HRP)-conjugated goat anti-horse IgG (H&L) (Abnova, Taiwan, China) was added to detect bound antibodies and incubated for 1 h at 37 °C. Subsequently, the plates were washed again using the same washing procedure and the peroxidase reaction was then visualized by incubating plates with TMB (IDEXX Laboratories, Westbrook, NJ, USA) for 15 min at room temperature. The reaction was stopped by adding 50 µL/well sulfuric acid (H_2SO_4 0.5 M (VWR International BV, Amsterdam, The Netherlands)) and optical densities (OD) were immediately measured at 450 nm using an ELISA microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). All serum samples were tested in duplicate. S/P values were calculated with the formula: S/P = (OD Sample-OD Negative control)/(OD Positive control-OD Negative control).

2.6. Statistical Analysis

The correlation between OD values scored with two ELISA formats was measured by the Pearson correlation coefficient using Graph Pad Prism, version 7. The discriminating power of the two different ELISA formats was analyzed by performing receiver operator characteristic (ROC) analysis with 231 sera, which 94 were negative (VNT < 8) and 137 were positive (VNT ≥ 8). The cut-off value, diagnostic specificity and sensitivity were determined by ROC analysis using Sigmaplot. A minimum specificity of 99% was chosen for the selection of cut-off values. Additionally, the reproducibility of assays was

evaluated by testing three samples with different OD values. Inter-assay coefficients of variation (CV) and intra-assay CV were determined testing each sample in triplicate on three different plates in three different runs and within the same plate, respectively.

3 Results

3.1. Determination of Neutralizing Antibodies

To identify equine sera containing ECoV-neutralizing antibodies, we screened a subset of 231 equine sera, composed of randomly selected serum samples from panels A–C, and all samples from panel D were screened in the VN assay. Of the 231 sera, 94 sera were tested as negative (titers < 8) and 137 positive samples (titers ranging from 8 to 4096).

Additionally, paired samples from 27 horses (n = 54, panel I) were tested in the VN assay. Twenty out of 27 sera collected from the first time point exhibit titers ranging from 12 to 2048. The convalescent serum samples were collected 21–28 days following the first round of sample collections, and all of them showed neutralization responses with titers ranging from 16 to 4096. Within these horses, seven of them showed seroconversion and 14 showed a significant (4-fold or greater: 2log2) increase in titer in the VN assay. To confirm the presence of ECoV specific IgG in Icelandic horses, 24 horse sera with positive ECoV S1 ELISA results (in panel H, S/P value > 0.5) were tested in ECoV neutralization assays. All of them had neutralizing antibodies with titers varying between 32 and 768.

3.2. Development of ECoV S1 ELISA

Besides the conventional wet ELISA format (wELISA) for general laboratory usage, a dry standardized ELISA format (dELISA) was also developed and validated to facilitate implementation as routine diagnostic method in different laboratories and possibly wider application as an ELISA kit. Both ELISA formats were developed for the detection of ECoV-specific antibodies in horse serum samples. The diagnostic performance of both ELISAs was evaluated using a subset of 231 horse sera with known VN results as described above. The Pearson correlation coefficient was calculated to assess the correlation between the OD values obtained with the two ELISA formats (Figure 1). Results indicate that OD values obtained with both ELISAs show a high degree of correlation, with correlation and regression coefficients close to 1 (R^2 = 0.939, regression coefficient = 0.9513, p < 0.0001). Thus, the performance of both ELISA formats is very similar.

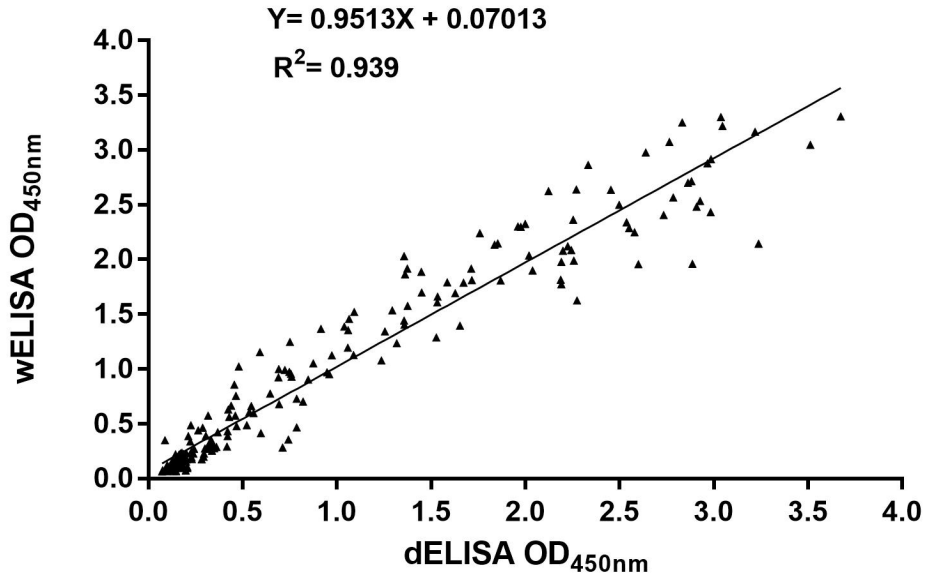

Figure 1. Correlation between optical density (OD) values obtained with wet format ELISA (wELISA) and dry format ELISA (dELISA).

Subsequently, the discriminating power of the wELISA and dELISA was evaluated via receiver operator characteristic (ROC) analysis. The ROC curves were plotted based on the previous classification of 231 sera into negative and positive by VN assays (Figure 2A,B). Then the optimal cut-off values, diagnostic specificity and sensitivity of both ELISA formats were determined by the established ROC curves. The ELISA results of the VNT-positive and negative samples are shown in Figure 2C,D. The diagnostic accuracy of both ELISA formats was considered to be high as the same area under the curve (AUC) values were observed (AUC = 0.985), with a relative sensitivity and specificity approximately 95% according to the Youden plot of wELISA and dELISA. Therefore, the test characteristics of both ELISA formats were assigned the same weight. In this study, a minimum specificity of 99% was chosen for the threshold of cut-off values for both ELISAs. Accordingly, the optimal cut-off for wELISA was an OD value of 0.35—for which, the sensitivity was 87% and the specificity was 99%. For dELISA, the test results were expressed as S/P values. A cut-off at an S/P value of 0.13 yielded a sensitivity of 85% and specificity of 99%, respectively.

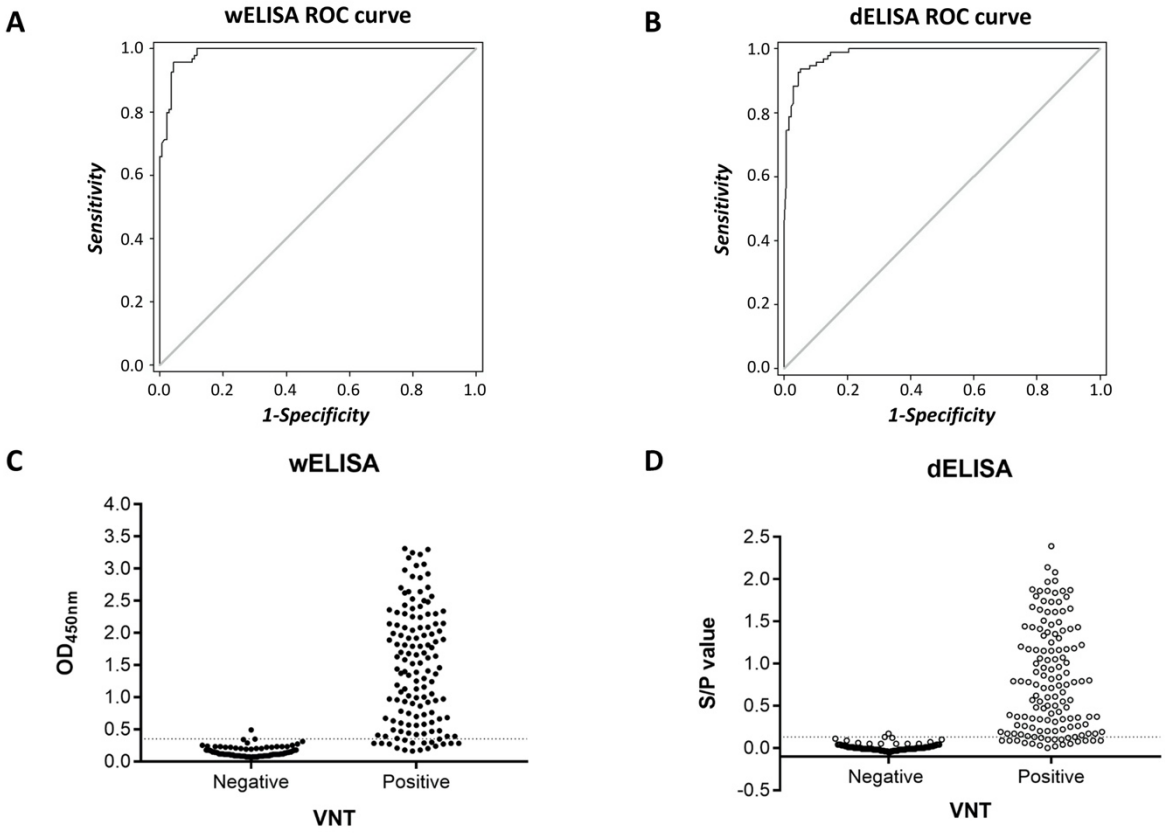

Figure 2. Receiver operating characteristic (ROC) analyses of equine coronavirus (ECoV) S1 ELISAs. ROC curves for wELISA (**A**) and dELISA (**B**) were plotted with positive (n = 137) and negative (n = 94) sera confirmed via VN assays. The area under the curve (AUC) is 0.985 for both ELISA formats. Distributions of wELISA (**C**) and dELISA (**D**) with confirmed sera are shown above. Calculated cut-off points are indicated by the vertical dashed lines. VN assays, virus neutralization assays; VNT, virus neutralization titer.

Furthermore, the inter- and intra-coefficient of variation (CV) of the three ECoV positive sera tested with both ELISA formats were lower than 12%. More specifically, the intra-assay CV of wELISA and dELISA ranged from 3.04% to 4.87% and from 5.4% to 7.7%, respectively, while the inter-assay CV of wELISA and dELISA varied from 4.9% to 10.26% and from 8.9% to 11.2%, respectively. Overall, these results indicate that the performances of both ELISA formats were very much equivalent and that the results of both ELISAs were strongly correlated to VN results.

3.3. Detection of Antibodies against ECoV in Horses during an Acute Outbreak

To determine the diagnostic performance of the ECoV S1 ELISA, 27 paired serum samples (panel I) collected from an acute ECoV outbreak were investigated by wELISA. The horses presented similar clinical signs as described in [27], and virus shedding was confirmed by qPCR analysis [7]. At the acute stage, 11 out of 27 horses were qPCR positive, while at the convalescent stage, this number had decreased to six. Serum samples were further validated by VN assay (Figure 3B; Table S1). Seven out

of 27 horses showed seroconversion, while another 14 horses showed a significant (4-fold or greater) increase in VNT. Performing the wELISA (see Figure 3A; Table S1), the same seven out of 27 horses showed seroconversion; acute phase sera were negative (OD value < 0.35) whereas the convalescent phase sera all had OD values greater than 1.00 (1.14–2.90). Thus, seroconversion rates calculated from wELISA and VNT showed a 100% correlation (Table S1). For the horses that showed a 4-fold or greater increase in VNT (n = 14), nine of the acute phase sera had positive OD values between 0.35 and 0.70 (2x background) and also a higher than 2 (n = 2) to 4 (n = 7) fold increase in the OD value in the convalescent serum. Five of the VNT positive paired serum samples had OD values of >0.70 (twice the background OD value) in the acute phase serum. Two of these samples with an OD value of 1.12 and 1.41 respectively in wELISA also showed a greater than 2-fold increase in OD value. The three VNT positive samples with less than 2-fold increase in OD values already had high OD values in the acute phase serum as well as high VNT (mean OD value = 2.51, mean VNT = 8.30). For the six horses that did not show a significant rise in VNT, five serum samples collected at the acute stage already had high antibody levels as shown by ELISA and neutralization assay (mean OD value > 2.6, mean VNT > 9, Table S1). Further, the Pearson correlation coefficient was calculated to assess the overall correlation between the OD values obtained with wELISA and VNT (log2 titers) from acute and convalescent-phase sera of the 27 horses (Figure S1). Results indicate that OD values and VNT show a good degree of correlation ($R^2 = 0.83$, $p < 0.0001$). These data support the use of the wELISA as a diagnostic tool in case of suspected ECoV outbreaks.

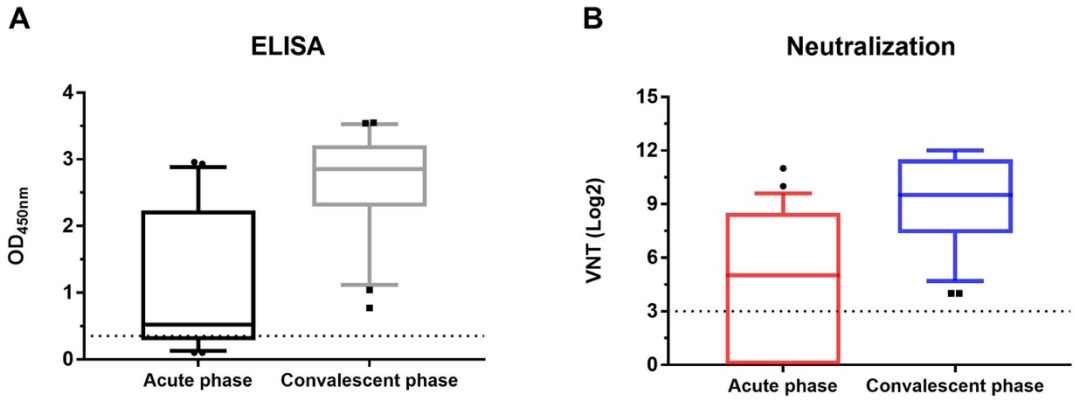

Figure 3. Antibodies response against ECoV from 27 horses during an acute outbreak. Boxplots show the ELISA reactivities (**A**) and VNT (**B**) of 27 horses from acute and convalescent-phase sera. Each cut-off is indicated by the dotted dashed line; VNT, virus neutralization titer.

3.4. ECoV Seroprevalence in Horses with Unknown ECoV Exposure

We further set out to determine the seroprevalence in horses with unknown ECoV exposure using the dELISA format. A total of 1084 serum samples (Table 1, panel A–H) were analyzed. With the exception

of panel D, all sera were from adult horses (older than 36 months). Seroprevalence varied from 25.9% (panel D) to 82.8% (panel C) among these eight serum panels. The lowest number of positive samples was found in panel D which contained young horses (6-30 months old, average age: 8.38 months (95% CI 6.975–9.785)). In the other four serum panels (panel A, B, E and F) from Dutch horses, the historical serum samples (panel G) and samples from Iceland (panel H) higher seroprevalences were found (59.2–82.8%).

Table 1. Prevalence of ECoV S1-reactive antibodies in equine sera used in this study.

Panel	Samples Source/Project Names	Collection Year	Country	Numbers of Samples	Numbers of ECoV-S1 Positive Samples	Seroprevalence (%)
A	West Nile virus (WNV) surveillance	2016	The Netherlands	167	128	76.60
B	Equine infectious anemia (EIA) surveillance	2016	The Netherlands	112	80	71.40
C	Export horses	2016	The Netherlands	99	82	82.80
D	Influenza surveillance	2015	The Netherlands	81	21	25.90
E	WNV surveillance	2015	The Netherlands	176	145	82.40
F	EIA surveillance	2015	The Netherlands	184	109	59.20
G	Equine herpesvirus 1 and 4 diagnostic serum panel	1990	The Netherlands	165	93	56.40
H	Horse sera from Iceland	2018	Iceland	100	62	62.00

4 Discussion

Since the beginning of the 21st century, ECoV infections have been reported in horses, causing fever and enteric diseases [4]. More recently, infections in adult horses were reported with clinical signs of fever, anorexia, lethargy and, less commonly, specific signs of diarrhea and colic [7,8]. Nevertheless, information regarding the circulation of ECoV in the equine population, especially in Europe, is still limited [6,28]. Serological studies are useful tools to investigate ECoV prevalence in horse populations. In the present study, our aim was to develop a simple and reliable method for antibody detection against ECoV that can be used for diagnostics and sero-epidemiological studies.

As compared to virus neutralization assays, the ELISA method has the advantage of being reproducible, potentially high-throughput and much less laborious. In our study, we set up an ECoV S1-based ELISA method in two complementary formats. The conventional wELISA format is for general laboratory usage with simplified, easy to perform coating procedures. On the other hand, coated plates of the dELISA format could be stored for a longer time period, making it ideal for transportation and kit development. We showed that both formats performed equally well, and their results correlated nicely. When comparing with the VN assay by ROC analysis, our ELISA method with both formats was shown to have high accuracy. In our current study we applied wELISA for the analysis of the paired outbreak samples, while the dELISA was further validated and used for the high-throughput screening of larger amount of serum samples.

We utilize ECoV S1 as the viral antigen for antibody detection in this study. The S1 chimeric protein was expressed in mammalian cells, and hence both the protein conformation and modification (e.g., glycosylation) are mimicking the S proteins on the surface of virus particles [29]. As the most divergent and immunodominant component of coronaviruses, S1 has been widely used in the development of methods for specific coronavirus serological studies [19,20,26,30]. Our findings validate that ECoV S1 is a highly suitable antigen for the detection of antibodies against ECoV showing very good agreement between the ELISA and VN assays. Recently, similar conclusions were also drawn for the role of MERS S1 in MERS serology [31].

With our wELISA method, we were able to analyze paired samples that were collected during an ECoV outbreak. In the virus neutralization assay seroconversion or a 4-fold or greater increase in ECoV antibody titers could be detected in sera of 21 out of 27 horses within weeks of the initial observation of clinical disease and detection of viral RNA in feces. Of these 21 positive horses 18 showed seroconversion or a 2-fold or higher increase in OD values in the wELISA. The three remaining VNT positive samples had high OD values already in the acute phase serum. Of the six ECoV negative paired samples five had high VN antibody titers and OD values already at the acute phase. This might be due to late sampling of these horses or previous exposure to ECoV (Table S1). This study confirms that the ECoV S1 ELISA is a useful diagnostic test for the demonstration of a potential ECoV outbreak and should be considered as a useful adjunct to investigation of fecal samples by qPCR.

We also determined the seroprevalence of serum samples collected from horses with unknown ECoV exposure via our dELISA. Results showed that the overall seroprevalence in the different cohorts tested is 25.9%–82.8%. These percentages are in agreement with the study performed by Hemida et al. [32], in which they detected coronavirus infections in horses in Saudi Arabia and Oman and they found that 74% of them had detectable neutralizing antibodies to ECoV. A lower percentage (9.6%) of positive animals was found in another ECoV seroprevalence study conducted in the USA [33]. Several factors might contribute to these differences in results. There is only limited information regarding ECoV prevalence in Europe including the Netherlands [6,28], and it is possible that the overall ECoV distribution differs between continents. Moreover, our study employs eukaryotically expressed ECoV S1 protein as coating antigens, while in the US study chimeric S2 protein expressed in Escherichia coli was used. The expression in mammalian cells guarantees a more native configuration of the protein, in particular of glycosylated antigens such as the coronavirus spike protein. Reports had shown that both coronavirus S1 and S2 subunit elicit antibody responses, but the level of immune responses triggered by them may differ [34,35]. Furthermore, the criteria for determining the cut-off value are different for the two studies. In our study we defined positive and negative samples on the basis of a VN assay, whereas the US study used negative qRT-PCR and absence of clinical signs as criteria to define horses

as ECoV negative. In this way, seropositive horses may have contributed to higher cut-off values and potentially a lower sensitivity of the assay.

In our study, we noticed differences in seroprevalence between young and adult horses. In the group of young horses (panel D, Table 1), the lowest seroprevalence was found. Young horses may initially be protected against ECoV infection by maternal antibodies and may become gradually more susceptible as maternal antibodies wane. The risk of becoming infected increases with age. This hypothesis is further supported by the age distribution of PCR-confirmed ECoV infection cases: foals (age 0–6 months) have the lowest infection rates, and the infection rate increases with age [10].

We also observed a significant percentage of seropositive horse serum samples collected back in 1990 (panel G, Table 1). ECoV-like viruses were detected in the 70s and 80s by electron microscopy in feces of horses with enteric disease, but virus isolation and characterization was not reported [36–39]. The history of ECoV presence, especially in Europe, is possibly much longer than currently understood [6]. Intriguingly, we noticed that Icelandic horses also are seropositive against ECoV (panel H, Table 1). Twenty-four serum samples showed high ECoV ELISA reactivity (S/P value > 0.5) and also had neutralizing antibodies with VNT varying between 32 and 768. The horse population of Iceland has been geographically isolated for more than 1000 years and is free from most common equine contagious diseases such as equine influenza, equine herpesvirus 1, strangles and equine viral arteritis [40]. To date, no prior studies of ECoV prevalence in horses from Iceland had been performed. This is the first evidence of the existence of ECoV infection in Iceland.

In conclusion, we developed a high-throughput, reliable and specific ELISA method to study humoral immune responses in horses against ECoV. With this method, we are able to perform the serodiagnosis of ECoV infection and assess the seroprevalence within horse populations in the future.

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Supplementary information

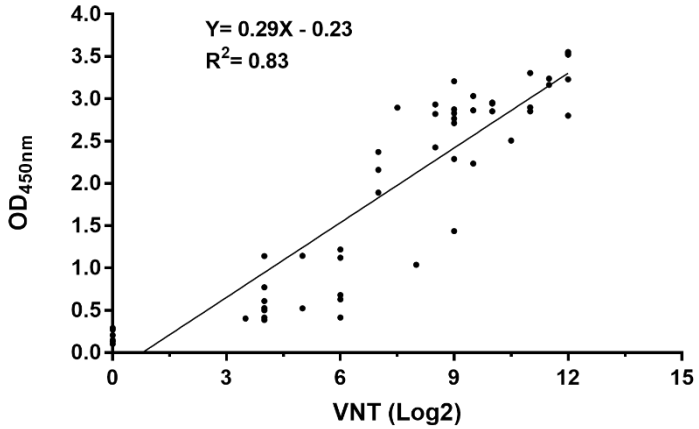

Figure S1. Correlation between the OD values obtained with wELISA and virus neutralization titers (VNT) of 27 horses from acute and convalescent-phase sera. Negative sera [VNT <8, (3log2)], are represented as 0 in the graph.

Table S1 Detection of antibodies to ECoV in equine serum samples during an ECoV outbreak in winter 2014

Acute phase						Convalescent phase			
Animal Name	**Date (month.day.year)**	**qPCR ECoV resutls**	**wELISA OD values**	**VNT ECoV (log2)**		**Date (month.day.year)**	**qPCR ECoV resutls**	**wELISA OD values**	**VNT ECoV (log2)**
1. Ramos	12.12.2014	+	0.15	0		01.07.2015	+	2.37	7
2. Utrillo	12.12.2014	+	0.50	4		01.07.2015	-	3.16	11.5
3. Julia	12.18.2014	-	0.52	5		01.07.2015	-	2.50	10.5
4. Sawyer	12.18.2014	+	2.23	9.5		01.07.2015	+	3.03	9.5
5. othello	12.18.2014	+	2.85	11		01.07.2015	-	3.54	12
6. Gio	12.18.2014	-	2.87	9		01.07.2015	+	3.55	12
7. Daisy	12.18.2014	+	0.27	0		01.07.2015	+	1.14	4
8. Diola	12.18.2014	+	1.44	9		01.07.2015	-	3.24	11.5
9. Reagan	12.18.2014	+	0.41	4		01.07.2015	-	0.77	4
10. Wencenza	12.18.2014	-	0.61	4		01.07.2015		1.22	6
11. Leiden	12.18.2014	+	2.42	8.5		01.07.2015	-	2.16	7
12. Grayton Beach	12.18.2014	-	2.93	8.5		01.07.2015	-	2.82	8.5
13. Nelson	12.18.2014	-	0.13	0		01.07.2015	-	2.29	9
14. Weltkaar	12.18.2014	-	0.38	4		01.07.2015	-	1.04	8
15. Broadway	12.18.2014	+	0.63	6		01.07.2015	-	3.30	11
16. Manhattan	12.18.2014	-	2.96	10		01.07.2015	-	2.86	9.5
17. Prism	12.18.2014	-	0.10	0		01.07.2015	-	2.83	9
18. Cover Girl	12.18.2014	-	0.21	0		01.07.2015	+	2.90	7.5
19. Parma	12.18.2014	+	1.12	6		01.07.2015	-	2.71	9
20. Henry	12.18.2014	+	2.77	9		01.07.2015	-	3.52	12
21. Antone	12.18.2014	-	1.89	7		01.07.2015	-	3.23	12
22. Abby	12.18.2014	-	0.40	3.5		01.07.2015	-	2.85	10
23. Sardi	12.18.2014	-	0.52	4		01.07.2015	-	2.94	10
25. Hello Daisy	12.18.2014	-	0.68	6		01.07.2015	-	3.21	9
27. Levi	12.18.2014	-	0.41	6		01.07.2015	-	2.80	12
28. Billie Jean	12.18.2014	-	0.10	0		01.07.2015	+	1.14	5
29. Flirt	12.18.2014	-	0.29	0		01.07.2015	-	2.90	11

Negative sera [VNT <8, (3log2)], are represented as 0 in the table

Marked yellow: animals showed seroconversion in virus neutralization titers (VNT)

Marked orange: animals with ≥4-fold increase in VNT

Chapter 4

Development and application of a toolbox for serological screening of SARS-CoV-2 infection in cats and dogs during the first COVID-19 wave in the Netherlands

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Abstract

The new coronavirus SARS-CoV-2 is thought to have emerged in humans after a spill-over event from an animal source and subsequently spread globally via efficient human-to-human transmission. Besides humans, SARS-CoV-2 can also infect many other animal species including cats and dogs. In the present study, we developed and validated a set of serological assays including ELISA and virus neutralization. Evaluation with samples from pre-COVID-19 and SARS-CoV-2-exposed cohorts confirmed the suitability of these assays for specific antibody detection. Furthermore, our findings also disqualify SARS-CoV-2 N protein as an antigen for serological screening of cat and dog samples. Applying these assays, five hundred sera each from domestic cats and dogs with unknown SARS-CoV-2 exposure collected between April and May 2020 were analyzed to assess the prevalence of antibodies of SARS-CoV-2 infections in Dutch cats and dogs. Our study shows that 0.4% (95% confidence interval, 0.01%-1.55%) of cats and 0.2% (95% confidence interval, <0.01%-1.24%) of dogs were seropositive at the time of sampling. We also applied an antigen adsorption assay to show that two betacoronavirus infections in dogs (SARS-CoV-2 and canine respiratory coronavirus) can be distinguished serologically. Overall, we present the development and validation of SARS-CoV-2 serological assays for cats and dogs, and we conducted the first serological study in the Netherlands. The general prevalence of antibodies to SARS-CoV-2 in the animal population with unknown SARS-CoV-2 exposure included in our study is low but supports the importance of continuous serosurveillance of SARS-CoV-2 in these companion animals.

Importance

SARS-CoV-2 is an emerging pathogen that is currently posing severe burdens on people's health and welfare worldwide. Due to reverse zoonosis, sporadic events of SARS-CoV-2 infection in companion animals like cats and dogs have been reported. To fully understand the implications of SARS-CoV-2 in these animals, accurate diagnostic methods for surveillance and epidemiological studies are needed. In the present report, the development and validation of specific SARS-CoV-2 serological assays is described and the results of screening of cat and dog samples of different cohorts with these assays are presented. Animals with likely SARS-CoV-2 exposure displayed substantial seropositivity rates, whereas the prevalence of antibodies in a set of samples from a general population of cats and dogs was low. This work expands the toolbox for seroprevalence assessments of SARS-CoV-2 in cats and dogs, and the results of our serological study support the necessity of continuous serosurveillance of SARS-CoV-2 in companion animals.

1 Introduction

A novel human coronavirus (HCoV), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), emerged in Wuhan, China in December 2019, and causes a severe pandemic of respiratory disease (COVID-19) [1,2]. Since its emergence, SARS-CoV-2 had spread to 222 countries and over 72.8 million infected cases were reported (https://www.who.int/emergencies/diseases/novel-coronavirus-2019; accessed December 18th, 2020), which occurred via human-to-human transmission and mostly affected elderly and immunocompromised people [3].

SARS-CoV-2 is a zoonotic virus able to infect several mammals under experimental condition [4]. Recently, transmission of SARS-CoV-2 from humans to cats and dogs has been reported, resulting in asymptomatic infections in dogs, and both symptomatic and asymptomatic infections in cats [5–9]. There is currently no evidence that pets play a role in the ongoing pandemic. Nevertheless, the close contact between owners and pets and the interaction between dogs and cats from different households raises the question about the role of these animals in SARS-CoV-2 transmission. So far, diagnosis of SARS-CoV-2 in pets is conducted by molecular assays such as real-time PCR. However, viral nucleic acid is only detectable within a limited timeframe post infection. Consequently, serological screening of SARS-CoV-2-specific antibodies in cats and dogs is important to get insight into the prevalence of this infection and possible modes of transmission (human-to-animal, animal-to-animal and animal-to-human).

In the present study, we developed SARS-CoV-2 specific serological assays. Serum samples were first tested with in-house ELISA based on SARS-CoV-2 S1 and receptor binding domain (RBD) proteins, and subsequently analyzed by virus neutralization assay using SARS-CoV-2 spike pseudotyped virus [10]. Utilizing these assay platforms, we conducted the first serosurveillance study of SARS-CoV-2 in cats and dogs of unknown SARS-CoV-2 exposure in the Netherlands. Our findings support the need of prolonged monitoring of SARS-CoV-2 infection in companion animals.

2 Materials and Methods

2.1. Serum Samples

Cat and dog serum samples collected in 2019 (pre COVID-19 cohort, n=45 each) were retrieved from the serum bank of our institute. Pre- and post-infection serum samples of feline coronavirus (FCoV) type I infected specific pathogen free (SPF) cats (n=9) were obtained from SPF cats infected with FCoV strain UU2 or RM in a previous study [11]. The SARS-CoV-2 exposed cohort consisted of 44 serum samples from stray cats roaming around SARS-CoV-2 positive mink farms [12], and one serum sample of a dog from a COVID-19 confirmed household. The 2020 cohort comprises domestic cat and dog serum or plasma samples (n=500 each: cat 119 serum and 381 plasma, dog 120 serum and 380 plasma)

that were sent to the University Veterinary Diagnostic Laboratory (UVDL) or the Veterinary Microbiological Diagnostic Center (VMDC) of Utrecht University for routine diagnostics between April and May 2020. Data on possible SARS-CoV-2 exposure of these animals was unavailable. All blood samples originated from veterinary clinics in the Netherlands and were evaluated for reasons independent to the study and collected via informed consent. All samples were stored at −20 °C until use and heat-inactivated at 56 °C for 30 min before usage.

2.2. Antigen preparation

Strep-tagged SARS-CoV-2 spike (S) subunit 1 (S1) and receptor-binding domain (RBD) proteins were produced in eukaryotic cells as described recently [10,13], and strep-tagged bovine coronavirus (BCoV) S1 (GB: P15777.1) and HCoV-229E S1 (GB: NP_073551.1) were cloned and produced similarly. SARS-CoV-2 nucleocapsid (N) protein was purchased from Sino Biological (Beijing, China). Mouse Fc-tagged FCoV type I S1 (GB: FJ938060.1), FCoV type II S1 (GB: AY994055.1) or BCoV S1 proteins were produced as described [14]. Vesicular stomatitis virus (VSV) pseudotyped with SARS-CoV-2 S protein (SARS2-VSV) was prepared as described recently [10] and titrated on Vero E6 cells.

2.3. Enzyme-Linked Immunosorbent Assay (ELISA)

Samples from all three cohorts were screened first with the indirect Enzyme-Linked Immunosorbent Assay (ELISA) against the different proteins, performed as previously described [14]. Briefly, high binding microtiter plates were coated with equal molar amount of protein (1 pmol per well after optimizing by checkerboard titration), diluted in phosphate buffer saline [PBS]) and blocked with blocking buffer (PBS containing 0.05% Tween-20 and 5% milk powder). A standard 1:50 dilution of serum samples or serial 2-fold dilutions of serum samples starting with 1:50 were next added to the wells. After one hour incubation at 37°C, plates were washed and subsequently incubated with horseradish peroxidase (HRP) conjugated secondary antibody (1:4000 for goat anti-cat IgG/HRP [Rockland Immunochemicals]; 1:6000 for goat anti-dog IgG/HRP [Cappel], diluted in blocking buffer) for one hour at 37°C. Peroxidase reaction was visualized by TMB substrate incubation (10 minutes at room temperature) and quenching with sulfuric acid. Optical densities (OD) were measured at 450 nm. For some of the 500 cats and dogs, serum as well as plasma was available. In our assay, no difference in OD values between available serum and plasma samples from the same animal was observed. Cut-off values were determined at 6-fold standard deviation above the mean value of reactivity of all negative serum samples from the pre COVID-19 cohort [13].

2.4. S1 adsorption assay

To prove that the infection with canine respiratory coronavirus (CRCoV), the endemic betacoronavirus infections in dogs can be distinguished serologically from an infection with SARS-CoV-2 [6,15], we designed an antigen S1 adsorption assay. Serum samples (20 µL each) were incubated with Strep-Tactin

sepharose (IBA) beads-conjugated with 20 µg S1 protein of SARS-CoV-2, BCoV or HCoV-229E for 3 hours at 4°C, followed by centrifugation at 11,000 x g for 30 min to remove S1-antibody complexes. Both mock-absorbed and protein-absorbed sera were two-fold serial diluted starting from a 1:50 dilution and further tested with S1 and RBD ELISA. IgG titers were expressed as the reciprocal of highest serum dilution resulting in OD values above the cut-off value.

2.5. Pseudovirus neutralization (pVN) assay

Pseudovirus virus neutralization (pVN) assay was carried out on Vero E6 cells in a 96-well plate. Samples (starting dilution at 1:8) were serial diluted 2-fold and mixed 1:1 with luciferase encoding VSV particles pseudotyped with S protein of SARS-CoV-2 (SARS2-VSV). Mixtures were pre-incubated at 37°C for 1 h and used for inoculation on cells. 24 hours post infection, the cells were lysed and relative luminescence units (RLU) of luciferase activity was determined as described recently [10]. RLU reduction rates of samples were calculated with the formula below:

$$Reduction\ Rate\ (\%) = \frac{RLU_{SARS2-VSV} - RLU_{mixture}}{RLU_{SARS2-VSV} - RLU_{blank}} 100\%$$

The sample neutralization titers were determined by the reciprocal of the highest dilution that resulted in >50% reduction of luciferase activity [pVNT (IC50 titer)]. Based on some aspecific inhibition of virus replication with SARS-CoV-2 negative samples at a dilution of 1:8, we set the cut-off at a dilution of ≥16 [16].

2.6. Statistical Analyses

All statistical analyses were performed using GraphPad Prism version 7.04 for Windows, GraphPad Software, La Jolla California USA. The Pearson correlation coefficient was calculated to determine the correlation between different ELISA OD and pVNT. The 95% confidence interval (95% CI) was determined by the modified Wald method.

3 Results

3.1. Pre COVID-19 cohort

Serum samples from the pre COVID-19 cohort were included as a specificity cohort and tested against SARS-CoV-2 S1 and RBD in an ELISA, and in a SARS-CoV-2 S VSV pseudovirus (SARS2-VSV) neutralization assay, to screen for potential cross-reactive antibodies elicited by endemic coronaviruses (CoVs) in cats and dogs. They are natural reservoirs of several CoVs, i.e. FCoV in cats, canine coronavirus (CCoV) and CRCoV in dogs [14,15,17]. FCoV type I S1 was used as an additional antigen to assess the reactivity of cat sera. For dog serology, the FCoV type II S1 (92.1% similar to S1 of CCoV) and BCoV S1 (95.7% similar to S1 of CRCoV) were taken along as proxy antigens for CCoV and CRCoV, respectively. As indicated in Figure 1, many sera were positive for FCoV and BCoV S1, but

all samples were negative for antibodies against SARS-CoV-2 S1 and RBD. Due to limited sample volumes, a selection of serum samples (n=34 for cat and n=24 for dog) was tested for SARS-CoV-2 S-bearing VSV pseudovirus (SARS2-VSV) neutralization, and all were tested negative (pVNT <16).

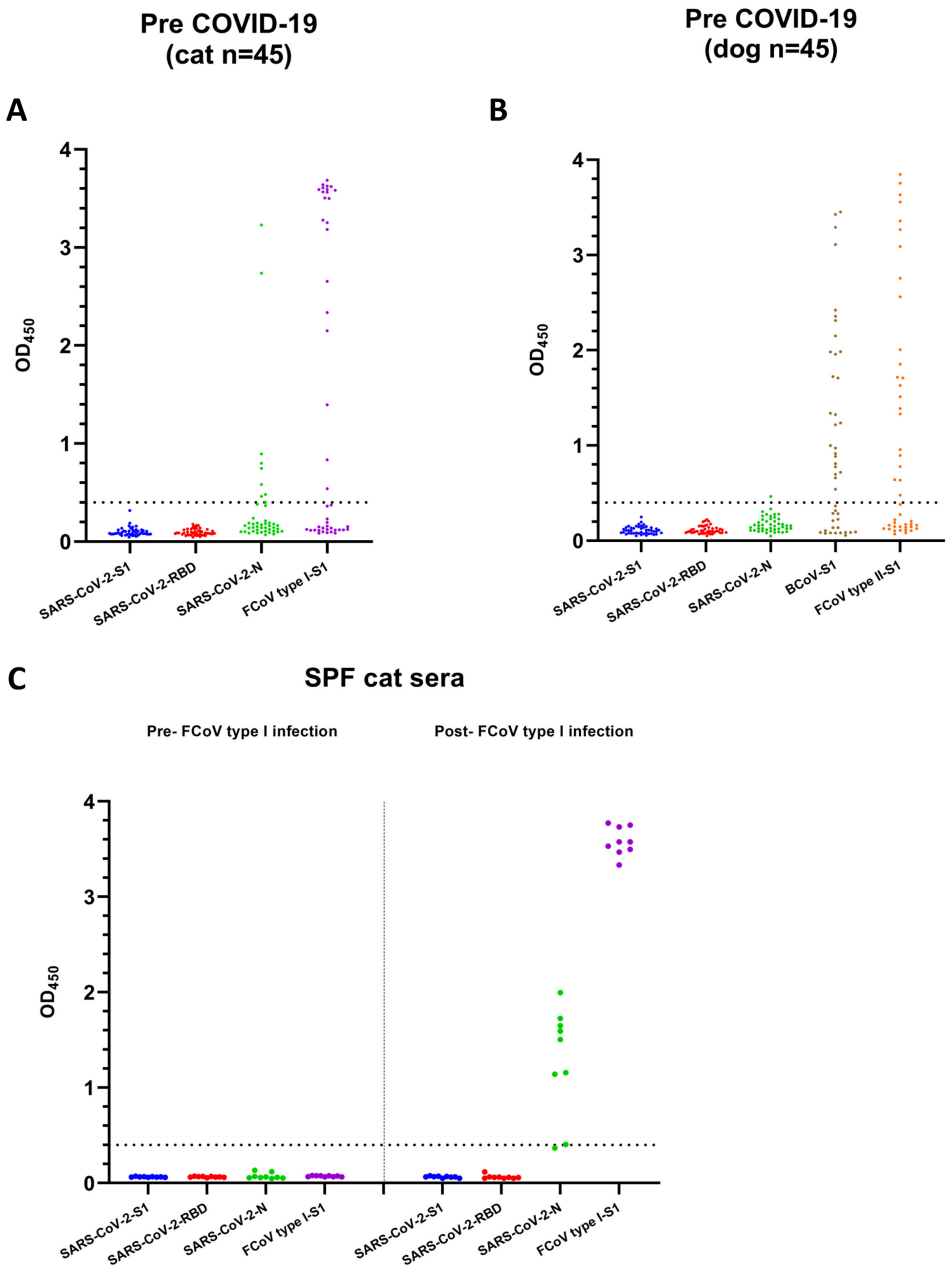

Figure 1. ELISA reactivities against different antigens of pre COVID-19 cat and dog serum samples and paired samples of FCoV type I infection. (A) ELISA reactivities of pre COVID-19 cat serum samples against SARS-CoV-2 S1, RBD, N and FCoV type I S1. (B) ELISA reactivities of pre COVID-19 dog serum samples against SARS-CoV-2 S1, RBD, N, BCoV S1 and FCoV type II S1. (C) ELISA reactivities of paired specific pathogen free (SPF) cat sera (left panel) and FCoV type I specific sera (right panel) to SARS-CoV-2 S1, RBD, N and FCoV S1 proteins determined by ELISA. The dashed lines in figures indicate the positive cut-off levels.

Meanwhile, 8 of the 45 (17.8%) pre-COVID cat sera and 1 of 45 (2.2%) of dog sera were positive in the SARS-CoV-2 N protein ELISA (Figure 1A and 1B). To explore this further, paired serum samples of SPF cats infected with FCoV were analyzed (Figure 1C). Serum samples from uninfected SPF cats were negative, while after FCoV infection 8 out 9 (88.9%) of the cats had developed antibodies reacting with SARS-CoV-2 N protein. All 9 samples were negative for antibodies against SARS-CoV-2 S1 and RBD. Compared to S1 and RBD proteins, the N protein is more conserved among CoVs (Supplementary Table 1), which might explain the cross-reactivity between FCoV and SARS-CoV-2 detected in our ELISA assays.

3.2. SARS-CoV-2 exposed cohort

We further tested sera of SARS-CoV-2 exposed stray cats living in the surroundings of SARS-CoV-2 positive mink farms [12]. These cats had access to the stables with cages in which the minks were housed. This feline cohort was expected to contain a higher number of SARS-CoV-2 positive samples because of close contact between the cats and mink. Also, a serum of a dog from a COVID-19 confirmed household was included together with the feline sera as a source of suitable samples for validation of our ELISA and pVN assay. In total, 11 out of 45 serum samples (24.4%; 95% CI, 14.1%-38.8%, 10 cats and the one dog) were positive in ELISA against both SARS-CoV-2 S1 and RBD, and 10 out of 45 samples (22.2%; 95% CI, 12.4%-36.5%) were reactive against SARS-CoV-2 N (Figure 2A). Notably, all S1 and RBD positive samples could neutralize SARS2-VSV infection, while N positivity and virus neutralization ability were not well associated (Figure 2B).

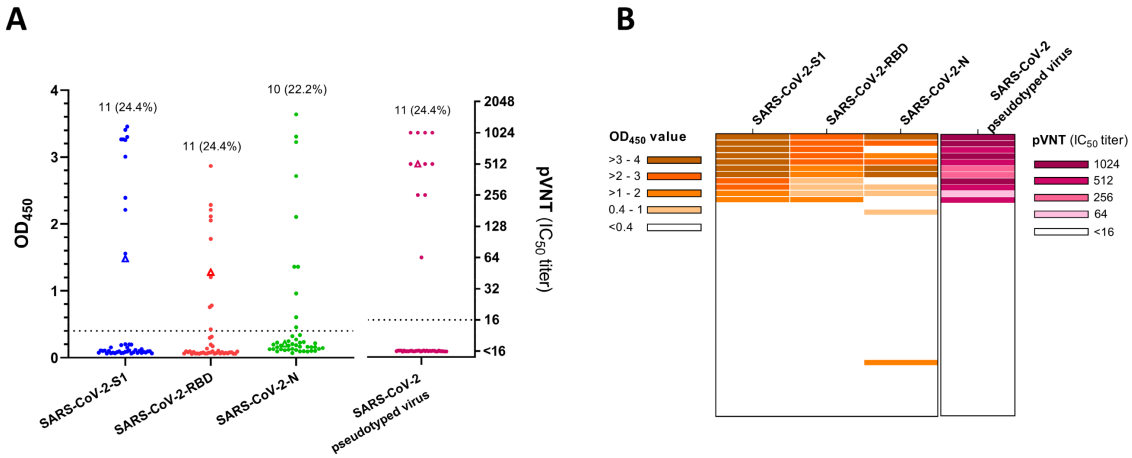

Figure 2. Serological analyses of cat and dog serum samples from SARS-CoV-2 exposed cohort. (A) ELISA against SARS-CoV-2 S1, RBD and N proteins and pVN analysis with SARS-CoV-2 pseudotyped virus. Cat serum samples (n=44) were indicated in dots and the dog sample (n=1) in triangle. (B) Combination of results tested by different assays expressed as a heatmap. Dashed lines show the positive cut-off levels.

OD values obtained with SARS-CoV-2 S1 and RBD ELISA showed a strong correlation with each other (R=0.95), and both correlated well with pVNT (R=0.87 for both; Figure 3A-3C). On the contrary, only poor correlation between OD values obtained with N ELISA and pVNT was observed (R=0.57; Figure 3D).

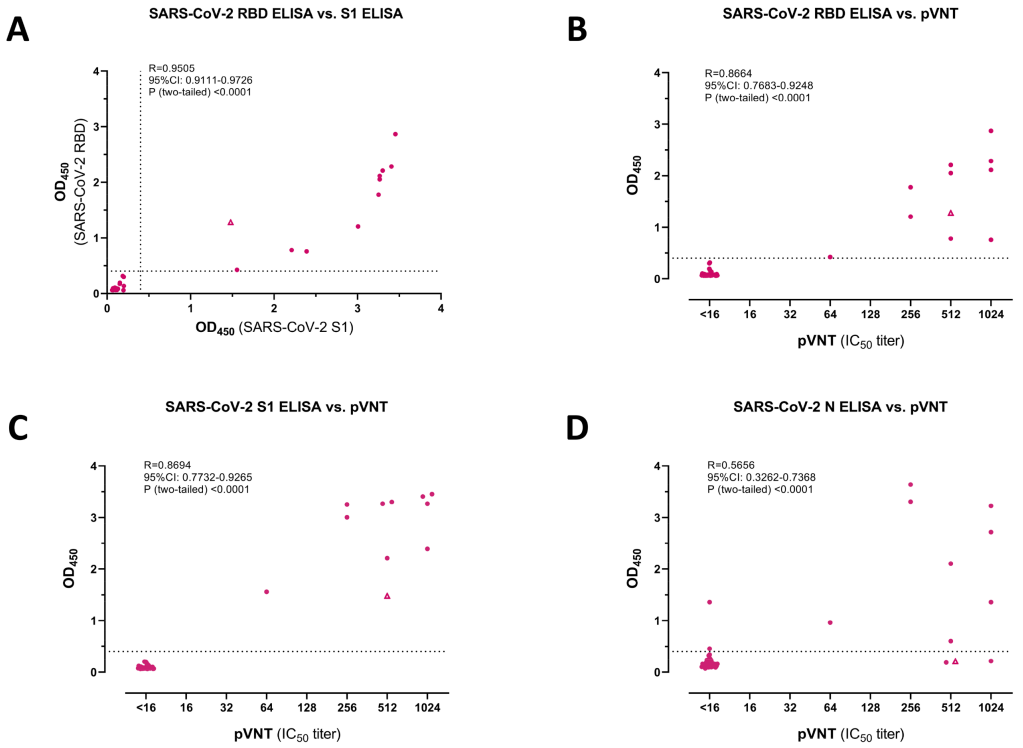

Figure 3. Pairwise correlation analyses of reactivities acquired from serological analyses of SARS-CoV-2 exposed cohort. The Pearson correlation coefficient was calculated to determine the correlation between the reactivities of RBD ELISA and S1 ELISA (A), or RBD ELISA with pVNT (B), S1 ELISA with pVNT (C) and N ELISA with pVNT (D). Cat serum samples (n=44) were indicated in dots and the dog sample (n=1) in triangle. Dashed lines show the positive cut-off levels.

3.3. SARS-CoV-2 seroprevalence in domestic cats

A total of 500 cat samples from the 2020 cohort were tested using SARS-CoV-2 S1 and RBD ELISA (Figure 4A and 4C). FCoV type I S1 was included as an additional antigen in the ELISA and 71% of cat samples were FCoV type I antibody positive (range of OD values: 0.42-4.01). Six cat samples were positive for both SARS-CoV-2 S1 and RBD, whereas an additional six samples were positive for RBD only (Figure 4C). Of these samples respectively six and five were also FCoV antibody positive. The results of the different tests are summarized in Supplementary Table 2. All SARS-CoV-2 S1 and/or RBD ELISA positive samples were tested by pVN assay, together with 50 randomly chosen samples that were negative in both S1 and RBD ELISA. Two samples that reacted with both SARS-CoV-2 S1 and RBD were able to neutralize SARS2-VSV infection, and all ELISA negative samples were also negative in pVN assay (Figure 4C, Supplementary Table 2). For serological assessment of infection

with many coronaviruses, virus neutralization assays are considered as the standard [18]. Here, based on results obtained with SARS-CoV-2 exposed animals, we defined a seropositive sample as any sample being ELISA positive for both SARS-CoV-2 S1 and RBD, and with pVNT ≥16. Samples that did not consistently reach diagnostic thresholds (ELISA positive for both S1 and RBD, but pVNT <16) were considered suspect (Supplementary Table 2). Accordingly, two out of five hundred (0.4%; 95% CI, 0.01%-1.55%) of domestic cat samples of unknown SARS-CoV-2 exposure had reached the diagnostic thresholds, and henceforth confirmed seropositive. Four sera were defined as suspect.

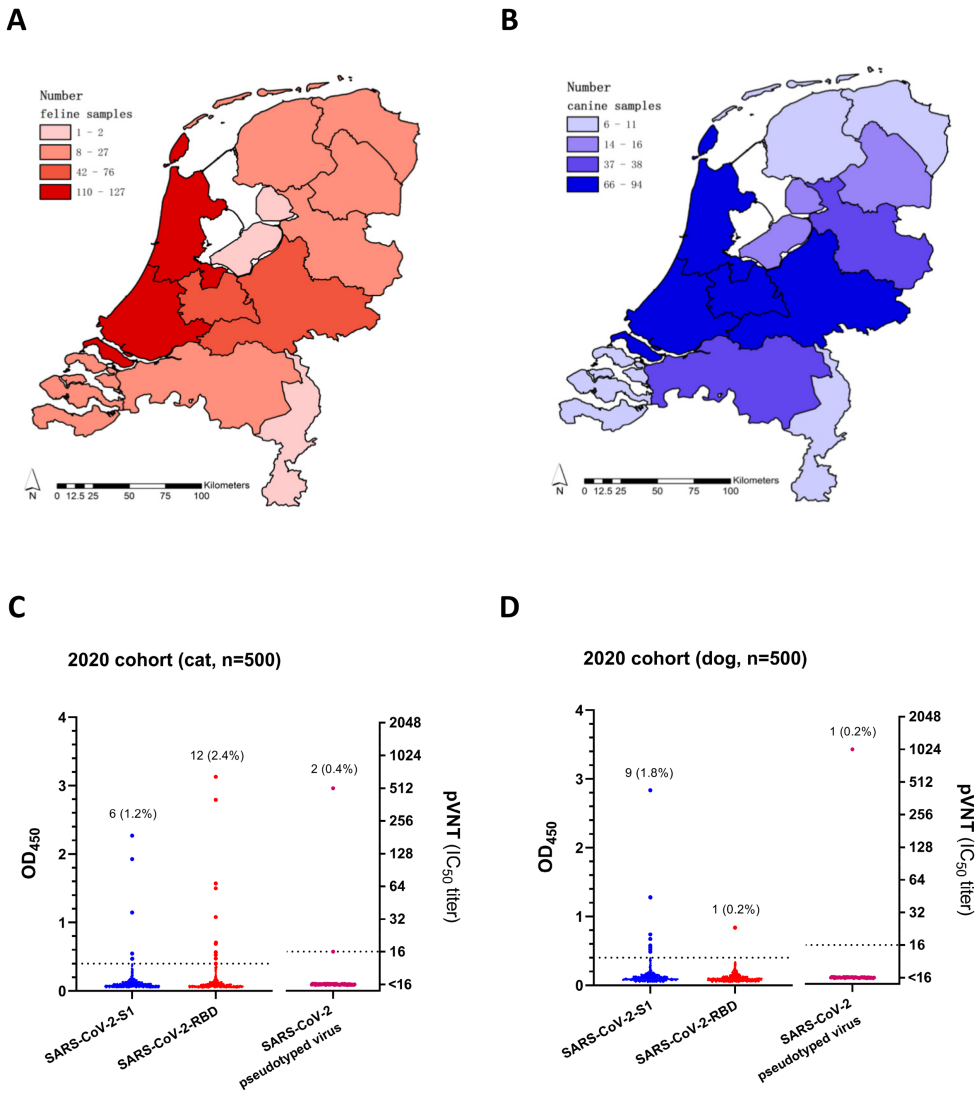

Figure 4. Geographical coverage and serological analysis of cat and dog samples of 2020 cohorts. (A, B) Distribution of cat (A) and dog (B) samples shown on the map of the Netherlands. Choropleth maps were produced using ARCGIS, version 9.3.1 (ESRI). (C, D) ELISA and pVNT analysis of cat (C) and dog (D) samples. Number and percentages of positive samples were indicated. The dashed lines show the positive cut-off levels.

3.4. SARS-CoV-2 seroprevalence in domestic dogs

We also tested 500 dog samples in the SARS-CoV-2 S1 and RBD ELISA (Figure 4B and 4D). FCoV type II S1 was included as an additional antigen and showed that 40.4% were FCoV type II S1 antibody positive (indicator of CCoV exposure). Nine samples were positive for SARS-CoV-2 S1, of which only one was positive for RBD (Figure 4D, Supplementary Table 2). Only the sample that reacted with both SARS-CoV-2 S1 and RBD was able to neutralize SARS2-VSV. Randomly chosen ELISA negative samples (n=50) were negative in pVN assay (Figure 4D, Supplementary Table 2). Thus, one out of 500 (0.2%; 95% CI, <0.01%-1.24%) of domestic dog samples of unknown SARS-CoV-2 exposure was considered seropositive.

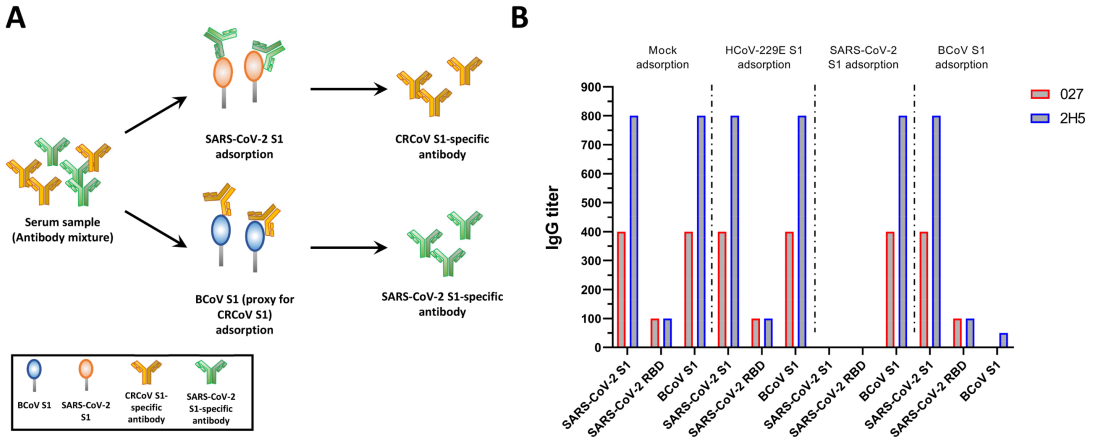

Figure 5. Corroboration of SARS-CoV-2 seropositivity in dog samples with adsorption assays. (A) Schematic depiction of antibody adsorption with S1 proteins. (B) ELISA reactivities of the two positive dog samples were determined against SARS-CoV-2 S1, RBD and BCoV S1 after mock adsorption or adsorption with HCoV-229E S1, SARS-CoV-2 S1 or BCoV S1 proteins. The two seropositive dog samples (027 and 2H5) are from SARS-CoV-2 exposed cohort and 2020 cohort respectively.

3.5. Adsorption assays confirmed SARS-CoV-2 specific antibodies in dog samples

The two seropositive dog samples (one from SARS-CoV-2 exposed cohort and another from 2020 cohort) also contained antibodies against CRCoV, which belongs to genus betacoronavirus as SARS-CoV-2 (Figure 5). BCoV is genetically very similar to CRCoV and therefore S1 of BCoV was used as antigen for screening of antibodies against CRCoV [19,20]. To corroborate SARS-CoV-2 seropositivity we performed antigen S1 adsorption assay with S1 proteins of SARS-CoV-2 or BCoV. HCoV-229E (from genus alphacoronavirus) S1 was taken along as a control. Where 229E S1 adsorption did not change ELISA reactivity of sera against SARS-CoV-2 and BCoV antigens, the SARS-CoV-2 and BCoV S1 adsorption specifically removed ELISA reactivity against the corresponding protein (Figure 5). These data confirmed that the ELISA reactivity against SARS-CoV-2 in these two dog samples is specific, in accordance with the screening of CRCoV positive pre COVID-19 dog samples described

above, which did not show cross-reaction with SARS-CoV-2 S1 in our ELISA assays.

4 Discussion

Since it was shown that SARS-CoV-2 can infect cats and dogs and transmission between animals can occur under experimental conditions [4], it is of general concern that the virus might spread in the cat and dog population and that animals might act as a reservoir with the possibility of animal-to-human transmission. Although so far the pandemic is driven by human-to-human transmission, it is important to know if domestic animals can play a role in maintenance and spread of SARS-CoV-2 infections in the future. This becomes especially important when SARS-CoV-2 transmission between humans starts to decrease and a virus reservoir in animals could become more relevant. To assess the risks, data on the prevalence of infections in different populations of animals are needed. For these studies verified serological assays that detect virus-specific antibody responses in cats and dogs are required. In the present study we build upon the assays used in human epidemiological studies, and validated ELISAs to detect SARS-CoV-2 S1 and RBD antibodies and virus neutralization using pseudotyped SARS2-VSV for screening cat and dog samples. Seropositivity was defined based on results of positive samples from the SARS-CoV-2 exposed cohort which recognized both SARS-CoV-2 S1 and RBD in ELISA and had a pVNT ≥16. Samples that did not consistently reach this diagnostic threshold (ELISA positive for both S1 and RBD, but pVNT <16) were considered suspected cases. We also showed that the N protein which is used in serological studies with human samples [13,21] lacks discriminating power. We found a poor correlation between the results of the N-ELISA and the pVNT and the S1 and RBD ELISA. Also several of the pre-COVID-19 samples were positive in the N-ELISA, most likely due to antigenic cross-reactivity between the SARS-CoV-2 and FCoV type I nucleocapsid proteins. These data validate SARS-CoV-2 S1 and RBD and disqualify N as antigen for serological screening of cat and dog serum samples. A similar phenomenon was also reported between porcine epidemic diarrhea virus and porcine transmissible gastroenteritis virus [22]. Therefore, the N protein cannot be used for serological screening of samples from cats and dogs.

To date, most studies focus on the molecular detection of SARS-CoV-2 in exposed animals, and virus detection is also used as the case definition for a confirmed case by OIE [23]. However, besides molecular detection serological studies are important to gain insight in the role of domestic animals in the epidemiology of the disease. In a recent molecular survey, no positive samples were detected from 4000 samples of companion animals that were submitted to a diagnostic lab for a respiratory PCR panel (cats, dogs and horses) [24]. However serological screening was not performed. In our study of samples from domestic animals with unknown SARS-CoV-2 exposure, we determined a prevalence of antibodies to SARS-CoV-2 in cats and dogs of 0.4% (95% CI, 0.01%-1.55%) and 0.2% (95% CI, <0.01%-1.24%) respectively, which is significantly lower than the prevalence rate of the endemic CoVs like FCoV and CCoV, and also lower than the seroprevalence estimate in Dutch human populations (2.7%-9.5%) at

the period of sample collection [25]. In our study we found also a much lower SARS-CoV-2 seropositivity than in domestic cats and dogs in northern Italy, where over 3% of samples were seropositive [26]. But these were all from animals living in SARS-CoV-2 positive households or in areas with high numbers of human infections. Such observations demonstrate that cats and dogs can acquire SARS-CoV-2 infection, but that the virus was not widely circulating in the population of Dutch cat and dogs included in this study at the time of sampling (April/May of 2020).

Virus neutralization assays are considered as the gold standard to assess immunity to many coronavirus infections based on their exceptional specificity [18]. Therefore, we defined a sample positive when the S1 and RBD ELISA were positive and confirmed by pVNT. In our screening, four cat samples were positive for antibodies directed against S1 and RBD in ELISA, but failed to neutralize SARS2-VSV infection and were defined as suspect as they did not reach our diagnostic threshold (supplementary table 2). This may be related to individual differences in developments of neutralizing antibodies. In humans with asymptomatic or mild infection of Middle East respiratory syndrome coronavirus or SARS-CoV-2, samples were reactive against antigens but failed to neutralize virus infection [18,27]. Moreover, 14 samples only reacted with S1 or RBD in ELISA and were defined as seronegative. Based on our results we conclude that the S1 and RBD ELISA is suitable for large-scale sero-epidemiological studies. Positive samples should next be confirmed by virus neutralization as a gold standard. Up to the present time, lack of knowledge on the kinetics of SARS-CoV-2 antibodies in cats and dogs is limiting the setup of validated serological assays, and systematic studies of development of antibody responses against different antigens in experimentally SARS-CoV-2 infected cats and dogs are needed.

Overall, we conducted the first serological study of SARS-CoV-2 infection in a specific cohort of domestic cats and dogs in the Netherlands. The general prevalence rate is low, indicating that cats and dogs are most likely to act as incidental hosts due to occasional SARS-CoV-2 spillover from humans. However continued sero-surveillance is important to monitor possible sustained transmission of SARS-CoV-2 infection in companion animals and a wider range of other animal species. This is especially important now the incidence of COVID-19 in humans is still increasing in several parts of the world.

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Supplementary information

Table S1. Percentage amino acid identity of canine and feline coronavirus spike and nucleocapsid proteins with SARS-CoV-2 proteins*

Genus	Virus	SARS-CoV-2			
		N	**S**	**S1**	**RBD**
Betacoronavirus	CRCoV	32.4	28.5	20.0	15.6
Alphacoronavirus	FCoV type I	29.0	24.0	16.8	7.7
	FCoV type II	27.8	25.3	17.7	8.9
	CCoV	28.0	25.1	16.9	8.9

* SARS-CoV-2, CRCoV, FCoV type I, FCoV type II, CCoV (GB: NC_045512.2, JX860640.1, FJ938060.1, AY994055.1, KC175341.1). Amino acid sequences were aligned by cluster W and pairwise identities were calculated with the needle method in the EMBOSS pairwise alignment algorithms program (http://www.ebi.ac.uk/Tools/psa/emboss_needle). N, nucleocapsid protein; S, spike protein; S1, spike S1 subunit; RBD, receptor-binding domain.

Table S2. Serological disposition of samples tested in different serological assays

Animal	Cohorts	SARS-CoV-2 S1 ELISA[a]	SARS-CoV-2 RBD ELISA[a]	pVNT[b]	No. samples	Conclusion
cat	SARS-CoV-2 exposed (total n=44)	+	+	+	10	Seropositive
		-	-	-	34	Seronegative
	2020 (total n=500)	+	+	+	2	Seropositive
		+	+	-	4	Suspect
		-	+	-	6	Seronegative
		-	-	- / NA	488	Seronegative
dog	SARS-CoV-2 exposed (total n=1)	+	+	+	1	Seropositive
	2020 (total n=500)	+	+	+	1	Seropositive
		+	-	-	8	Seronegative
		-	-	- / NA	491	Seronegative

[a] ELISA OD value ≥ cut-off value (0.4) is positive result (+), ELISA OD value < cutoff value (0.4) is negative result (-);
[b] pVNT, the neutralization titers of samples determined by the reciprocal of the highest dilution that resulted in >50% reduction of luciferase activity in pseudovirus virus neutralization. pVNT ≥ cut-off (16) is positive result (+), pVNT < cut-off (16) is negative result (-). NA, not applicable

Chapter 5

Serological Screening of Influenza A Virus Antibodies in Cats and Dogs Indicates Frequent Infection with Different Subtypes

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Abstract

Influenza A viruses (IAVs) infect humans and a variety of other animal species. Infections with some subtypes of IAV were also reported in domestic cats and dogs. In addition to animal health implications, close contact between companion animals and humans also poses a potential risk of zoonotic IAV infections. In this study, serum samples from different cat and dog cohorts were analyzed for IAV antibodies against seven IAV subtypes, using three distinctive IAV-specific assays differing in IAV subtype-specific discriminatory power and sensitivity. Enzyme-linked immunosorbent assays against the complete hemagglutinin (HA) ectodomain or the HA1 domain were used, as well as a novel nanoparticle-based, virus-free hemagglutination inhibition (HI) assay. Using these three assays, we found cat and dog sera from different cohorts to be positive for antibodies against one or more IAV subtypes/strains. Cat and dog serum samples collected after the 2009 pandemic H1N1 outbreak exhibit much higher seropositivity against H1 compared with samples from before 2009. Cat sera, furthermore, displayed higher reactivity for avian IAVs than dog sera. Our findings show the added value of using complementary serological assays, which are based on reactivity with different numbers of HA epitopes, to study IAV antibody responses and for improved serosurveillance of IAV infections. We conclude that infection of cats and dogs with both human and avian IAVs of different subtypes is prevalent. These observations highlight the role of cats and dogs in IAV ecology and indicate the potential of these companion animals to give rise to novel (reassorted) viruses with increased zoonotic potential.

1 Introduction

Influenza A viruses (IAVs) are enveloped, negative-sense segmented RNA viruses that belong to the *Orthomyxoviridae* family. They cause seasonal epidemics, pandemics and sporadic zoonotic infections [1]. Wild aquatic birds are the natural host reservoir, but IAVs have been isolated, besides from humans, from many other species, including cats, dogs, horses, pigs, mink, ferrets, foxes, marine mammals and domestic birds [2]. Thus, IAVs can easily cross the species barrier to infect new species. IAVs contain two glycoproteins expressed on the viral membrane, the hemagglutinin (HA) and the neuraminidase (NA). So far, 18 HA and 11 NA subtypes have been identified from IAVs circulating in birds and mammals, which through reassortment give rise to many viral subtypes with different HA/NA combinations [3–6]. In addition, IAVs often exhibit within-host genetic diversity because of their high mutation rate, efficient replication and large virus population sizes [7]. So far, IAV surveillance is mostly limited to birds, humans and swine. However, in view of the large number of mammalian species in which IAVs have been found and the large zoonotic potential of IAVs, surveillance should not be restricted to these few species.

Domestic cats and dogs are increasingly being recognized as hosts of IAVs. Previous studies have confirmed isolation of different avian and human IAVs of different subtypes in cats, including human-derived pandemic H1N1 (H1N1pdm09), which emerged in 2009 [8], and avian-derived H7N2, which caused an outbreak in a shelter in the USA in 2016/2017 [9]. The latter led to the first confirmed case of IAV transmission from cat to human [10]. Dogs are also susceptible to different IAVs. IAV outbreaks in dogs have been reported for equine-derived H3N8 viruses in 2004 [11] as well as for avian-derived H3N2 viruses [12,13]. Occasional spillover events of other IAV subtypes (H1N1pdm09, avian H5N1 and H5N2) to dogs have also been reported [14]. Notably, the majority of IAV infections in cats and dogs were found in Asia and North America, while in Europe only infections with H1N1pdm09, H3N8 and H5N1 have been reported [15–19]. These observations stress the potential role of cats and dogs in IAV circulation and the importance of IAV surveillance in these domestic animals.

Being the major surface glycoprotein of influenza virus, HA mediates binding of the virus to cell surface receptors and virus-cell fusion. It is also the prime target for neutralizing antibodies [20–22]. HA is a homotrimer, with each protomer comprised by two functional interdependent subunits, HA1 and HA2. Of note, the immunodominant globular head domain of HA formed by the central part of HA1, which contains the receptor-binding site (RBS), is the most variable IAV antigen. The more conserved and immune-subdominant stalk domain of HA is formed by the HA2 subunit along with N- and C-terminal regions of the HA1 [20,22]. Antibodies to the head domain of HA can be strongly neutralizing, but are mostly subtype or even strain specific, whereas antibodies against the stalk domain often show cross-reactivity within and across HA subtypes [23].

Serological screening can be used to support clinical diagnosis of IAV infection, herd immunity profiling, and monitoring of vaccine compliance. Moreover, it poses a foundation for seroprevalence-based epidemiological studies. Commonly used serological methods include hemagglutination-inhibition (HI) assays, which measure antibody titers by inhibition of the agglutination of erythrocytes, as well as enzyme-linked immunosorbent assay (ELISA) and virus neutralization (VN) assay that assess the presence of neutralizing antibodies. Incongruences between these different IAV serological assays have been reported [24,25]. VN and HI assays are generally recognized as "golden standards" but require culturing of potentially dangerous viruses. In addition, conventional HI, while being highly subtype specific, is relatively insensitive when detecting antibodies in animal and human sera [26,27].

In the present study, we developed a pipeline of serological assays which allow broad or specific analysis of IAV-specific antibody responses. In this pipeline, serum samples are tested first with HA- and HA1-specific ELISAs, and subsequently analyzed by nanoparticle-based, virus-free HI assays. Our study shows seroprevalence of antibodies to IAVs of both avian and human origin in European cats and dogs, which underscores the potential role of these domestic animals as IAV "mixing vessels".

2 Materials and Methods

2.1 Serum samples

Sheep polyclonal reference antisera (09/142 and 03/212) against purified HA of A/California/7/09 (Anti-$H1_{2009}$) and A/Wyoming/03/03 virus (Anti-$H3_{N2}$) were provided by the National Institute for Biological Standards and Control (London, UK). Goat polyclonal reference antiserum (NR-34586) to the HA of A/duck/Shantou/1283/2001 (Anti-$H3_{N8}$) and rabbit polyclonal reference antiserum (NR-48765) to the HA of A/Shanghai/1/2013 (Anti-$H7_{N9}$) were provided by Biodefense and Emerging Infectious Research Resources Repository (BEI Resources, Washington, DC). Chicken polyclonal antiserum against H5N8 A/Chicken/ Netherlands/SP00213/2017 (Anti-$H5_{N8}$) or H9N2 A/Chicken/Saudi Arabia/SP02525/3AAV/2000 (Anti-$H9_{N2}$) was provided by GD Animal health Deventer, the Netherlands. Seronegative normal goat serum (Invitrogen, Carlsbad, CA), normal rabbit serum (Invitrogen) and mock-vaccinated chicken serum from previous study [28] were included as negative controls.

For the serological screening, a total of 321 feline samples and 222 canine samples were included. Feline samples were obtained from three different cohorts: samples send to the University Veterinary Diagnostic Laboratory of Utrecht University before 2009 (serum samples, n=68) and in 2019 (plasma samples, n=131), and samples from abandoned or stray cats at shelters across the Netherlands of 2016 (serum samples, n=122). Canine samples were collected from two cohorts: samples collected before 2009 (serum samples, n=68) and between May and July 2019 (serum samples, n=154). All samples were collected for diagnostic purposes independent to this study. In addition, sera of specific pathogen

free (SPF) cats and dogs shown also to be negative for IAV were included as negative controls. All serum samples were stored at -20°C until tested.

2.2 Cells and viruses

Human embryonic kidney 293 cells stably expressing the SV40 large T antigen (HEK-293T), N-acetyl glucosamine transferase I-deficient HEK293S GnTI$^-$ cells [29] and Type II Madin-Darby canine kidney (MDCK-II) cells were maintained in Dulbecco modified Eagle medium (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (Bodinco, Alkmaar, The Netherlands), penicillin (100 IU/mL), and streptomycin (100 µg/mL).

Influenza virus A/Netherlands/602/2009 (H1N1) [30] was propagated in MDCK-II cells as described previously [31] and stored at −80°C until use. Prior to the use in hemagglutination/hemagglutination inhibition assays, virus was inactivated via UV radiation using UV Stratalinker 1800 (Stratagene) on 50,000 µJ.

2.3 Constructs design, protein expression and purification

Expression constructs of recombinant HA proteins are shown schematically in Figure 1. Human codon-optimized HA ectodomain (amino acids 24 to 530; H1 numbering) encoding cDNAs (GenScript, USA) of A/California/04/2009 (H1N1) (GenBank accession no.: ACS45035.1, referred to as $H1_{2009}$), A/Kentucky/UR06-0258/2007 (H1N1) (GenBank accession no.: ABX58635.1, referred to as $H1_{2007}$), A/Fujian/411/2002 (H3N2) (GenBank accession no.: AFD64223.1, referred to as $H3_{N2}$), A/canine/Florida/242/2003 (H3N8) (GenBank accession no.: ABA39842.1, referred to as $H3_{N8}$), A/Eurasian wigeon/Netherlands/1/2014 (H5N8) (GenBank accession no.: AKH60771.1, referred to as $H5_{N8}$), A/Anhui/1/2013 (H7N9) (GISAID isolate EPI439509, referred to as $H7_{N9}$), A/turkey/England/13437/2013 (H9N2) (GenBank accession no.: ALR82074.1, referred to as $H9_{N2}$) were cloned into a pFRT/pCD5 expression plasmid as described previously [32,33]. Briefly, the HA gene was cloned in an expression vector in frame with a sequence encoding for a CD5 signal peptide, a GCN4-isoleucine-zipper trimerization (GCN4) [34] and a Strep-tag II (ST) for purification (WSHPQFEK; IBA GmbH, Göttingen, Germany). In order to produce HA ectodomain trimers (HA-SpyTag) to be conjugated to its protein partner SpyCatcher, the constructs of HA were designed as above, with the addition of a sequence encoding a short (13-residue) SpyTag between GCN4 domain and ST encoding sequences [35]. To generate the head domain HA1 monomer (amino acids 24-320; H1 numbering), the sequence encoding the corresponding HA1 domain was cloned into pFRT expression vector flanked by the sequence encoding CD5 N-terminal signal peptide and ST.

HA and HA1 expression plasmids conjugated to polyethyleneimine (Polysciences, Inc., Warrington, PA) were transfected into HEK293T cells to produce soluble proteins for ELISA assays. HA-SpyTag-

encoding plasmids were expressed in HEK293S GnTI$^-$ cells instead of HEK293T cells, to increase the affinity of HA for its receptor [36]. At 6 to 7 days posttransfection, cell supernatants containing soluble HA and HA1 proteins were harvested, and proteins were purified using Strep-Tactin sepharose beads according to the manufacturer's instructions (IBA). Purified proteins were quantified by Nanodrop spectrophotometry (Thermo Fisher Scientific Inc., Waltham, MA) and analyzed on an SDS-PAGE gel under reducing conditions and visualized with GelCodeBlue stain reagent (Thermo Fisher Scientific). Purified proteins were stored at −80 °C until further usage.

2.4 Expression and purification of SpyCatcher-mi3 nanoparticles

pET28a-SpyCatcher-mi3 is a kind provided by Mark Howarth (Addgene plasmid, catalog no. 112255; RRID: Addgene_112255). The pET28a expression plasmid was transformed into E.coli BL21 (Agilent) similarly as described previously [37]. After 16h incubation at 37 °C, a single colony was picked into a starter culture LB medium from LB-Agar plate containing 50 μg/mL kanamycin, and incubated overnight at 37 °C with shaking at 220 rpm. Next, the entire 10-ml starter culture LB was diluted into 1 liter of LB culture medium, followed by incubation at 37 °C with shaking at 200 rpm. The cultures were induced with 0.5 mM IPTG until A_{600nm} reached 0.8 and grown for further 16 to 20h at 22 °C with shaking at 200 rpm. The pellet derived from 500ml culture was resuspended in 10 ml lysis buffer [25 mM Tris-HCl pH 8.5, 150 mM NaCl, 0.1 mg/mL lysozyme, 1 mg/mL complete mini EDTA-free protease inhibitor (Sigma-Aldrich) and 1 mM phenylmethanesulfonyl fluoride (Sigma-Aldrich) at 4 °C] and rotated at 25 °C for 1 h. After sonication on ice the lysis was spun down at 14,000g for 30min at 4 °C. For SpyCatcher-mi3 nanoparticle (NP) purification, Capture Select C-tag Affinity Matrix (Thermo Fisher Scientific) was added and incubated for 1h at 4 °C on a tube roller. NPs were then eluted with elution buffer (20 mM Tris-HCl pH 7.4 with 2 M $MgCl_2$ at 4 °C) according to the manufacturer's instructions. All eluted NPs were pooled and concentrated by 100 kDa MWCO Vivaspin ultrafiltration unit (Sartorius). NPs were then aliquoted and stored at −80 °C until further usage.

2.5 Western blot analysis

To assess the antigenicity of the expressed HA and HA1 proteins, 1 μg of purified HA or HA1 was loaded on a SDS-PAGE gel and subsequently electroblotted onto a polyvinylidene difluoride membrane. Membranes loaded with all HA or HA1 proteins were then incubated separately with reference antisera at 1: 200 dilution at room temperature for 1h. The antigen-antibody interaction was detected with horseradish peroxidase (HRP)-conjugated secondary antibody (1: 4000 for rabbit anti-goat/sheep immunoglobulins/HRP and swine anti-rabbit immunoglobulins/ HRP from Dako Agilent (Santa Clara, CA); 1: 4000 for goat anti-chicken immunoglobulin G /HRP from Southern Biotech Associates Inc. (Birmingham, AL)) and visualized by using Pierce ECL Western blotting substrate (Thermo Fisher) according to the user manual.

2.6 Enzyme-Linked Immunosorbent Assay (ELISA)

High binding microtiter plates (Greiner Bio-one BV, Alphen aan den Rijn, The Netherlands) were coated overnight at 4 °C with 1 µg/ml HA protein or 2 µg/ml HA1 protein (100 µL per well, diluted in phosphate buffered saline (PBS, pH 7.4)). After three washes with washing buffer (PBS containing 0.05% Tween-20), the plates were blocked for 2 h at 37 °C with blocking buffer (PBS containing 5% milk powder (Protifar, Nutricia, Zoetermeer, The Netherlands), 0.05% Tween-20). Protein coating efficiency was assessed by binding of StrepMAB-Classic (Strep-tag II specific monoclonal antibody) conjugated to horseradish peroxidase (IBA) in a direct ELISA. To determine the half maximal effective concentration (EC_{50}) of each reference antiserum against its homologous HA and HA1 proteins, 2-fold serial dilutions of serum samples were tested in an indirect ELISA format. Serum samples were diluted in blocking buffer (starting from 1: 50) and incubated in HA/HA1 coated plates at 37 °C for 1 h. After washing, plates were further incubated with diluted HRP-conjugated secondary antibody using the same concentration as for western blot analysis described above at 37 °C for 1 h. The peroxidase reaction was then visualized by adding TMB Super Slow One Component HRP Microwell Substrate (BioFX, Surmodics IVD, Inc., Eden Prairie, MN) for 10 min. Reaction was then quenched with 12.5% sulfuric acid and optical densities (OD) were measured at 450 nm. The OD values were plotted against dilution ratios, and EC_{50} of each reference antiserum against the homologous HA/HA1 was calculated by using 4 parameter logistic regression (4PL) curve fitting method (GraphPad Prism version v7.04) and expressed as dilution ratios. Experiments were performed in triplicate.

For the screening of serum samples, indirect ELISAs with different HA or HA1 proteins as antigens were conducted. In brief, reference antisera at EC_{50} or cat and dog sera at 1:200 dilution were incubated for one hour in HA/HA1 coated plates, and after washing the plates were further incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (reference antisera as described above; 1:4000 for goat anti-cat IgG/HRP (Rockland Immunochemicals, Inc., Pottstown, PA); 1:6000 for goat anti-dog IgG/HRP (Cappel, Costa Mesa, CA), followed by TMB development and sulfuric acid quenching. Seronegative goat, rabbit and chicken sera and sera from SPF cats and dogs were taken as negative controls to determine the cut-off values. Sera with OD values higher than 5-fold of the OD value of the corresponding negative sera were considered positive. ELISA results are expressed as ratios of OD_{450} value of the analyzed sera and the positive cut-off values (OD_{450} value of serum/positive cut-off value) [38,39]. All seven HA or seven HA1 proteins were coated on the same ELISA plates, making it easy to screen and compare the OD values of individual sera in one assay.

Part of the serum samples were also analyzed with a commercial competitive nucleoprotein ELISA (ID screen influenza A antibody competition, IDvet, Grabels, France) according to the manufacturer's user manual.

2.7 Hemagglutination and hemagglutination inhibition assays with HA-conjugated SpyCatcher-mi3 nanoparticles

Purified SpyCatcher-mi3 nanoparticles were incubated with 1.5-fold molar excess of HA-SpyTag (a single HA-SpyTag for conjugating homotypic nanoparticles) for 36 h at 25 °C in reaction buffer (25 mM Tris-HCl pH 8.5, 150 mM NaCl, pH 8.5). Coupling efficiency was analyzed by reducing SDS-PAGE gel stained with GelCode Blue (Thermo Fisher Scientific). The HA-NPs were then twofold serially diluted and mixed 1:1 with human erythrocytes (0.5% in PBS). Hemagglutination was assessed after 2h incubation on ice, and hemagglutinating units (HAU) were calculated for each HA-NP.

To study the hemagglutination inhibition ability of serum samples, HI assays were carried out according to the World Health Organization *Manual on Animal Influenza Diagnosis and Surveillance* [40]. Before analysis, samples were first treated with receptor-destroying enzyme (RDE) prior to reaction with HA-NPs. Serum samples were mixed with RDE (Denka Seiken Co., Ltd., Tokyo, Japan) in a 1: 3 ratio. The serum-RDE mixture was incubated at 37 °C for 16 h, followed by RDE inactivation at 56°C for 30 min. Treated sera were then 2-fold serially diluted and mixed 1:1 with PBS containing 4 HAU of HA-NPs. The mixtures were incubate at room temperature for 1 h, and then mixed 1:1 with human erythrocytes (0.5% in PBS). Hemagglutination inhibition was recorded after 2 h incubation on ice. The HI titer of a serum sample is expressed as the reciprocal of the highest serum dilution still showing inhibition of hemagglutination. Reference antisera, cat and dog sera and negative-control sera were tested in the same fashion as described above. Serum samples with an HI titer ≥ 1:20 were considered to be seropositive.

2.8 Statistical Analyses

All statistical analyses were performed using GraphPad Prism version v7.04 for Windows, GraphPad Software, La Jolla CA. The 95% confidence interval (95% CI) was computed by the modified Wald method. Cohen's kappa values were determined as a measure of the overall agreement. A chi-square test was used to assess the difference in the prevalence between two groups, and a p value of <0.05 was considered statistically significant.

3 Results

3.1 Conceptual design of ELISA and HI assays for serological screening

We devised a combination of assays including HA- and HA1-specific ELISAs and hemagglutination inhibition (HI), which differ in the number and nature of epitopes that are targeted. As a result, they differ in the breadth of antibody detection, theoretically ranging from broad to specific for different IAV subtypes and strains (Figure 1A). Using trimeric HA protein in ELISA allows detection of most epitopes present in both the variable HA1 and conserved HA2 subunit. Switching from HA to HA1, the range of detection will be narrowed down to HA1-specific epitopes. The HI assay only focuses on

epitopes surrounding the RBS, antibody binding to which will interfere with HA-receptor interactions (Figure 1A).

To allow evaluation of antibody responses against relevant IAV strains, we included HAs of representative IAV strains in our screens (Figure 1B). Our library contains HAs of IAV strains that have been found in humans, cats and/or dogs, such as H1 (human H1N1 circulating before and after 2009 ($H1_{2007}$ and $H1_{2009}$, respectively), H3 (human $H3_{N2}$ and canine $H3_{N8}$) as well as HAs of important avian IAV subtypes, $H5_{N8}$, $H7_{N9}$ and $H9_{N2}$. Collectively, these different HAs cover a large genetic and thus antigenic space (Figure 1B).

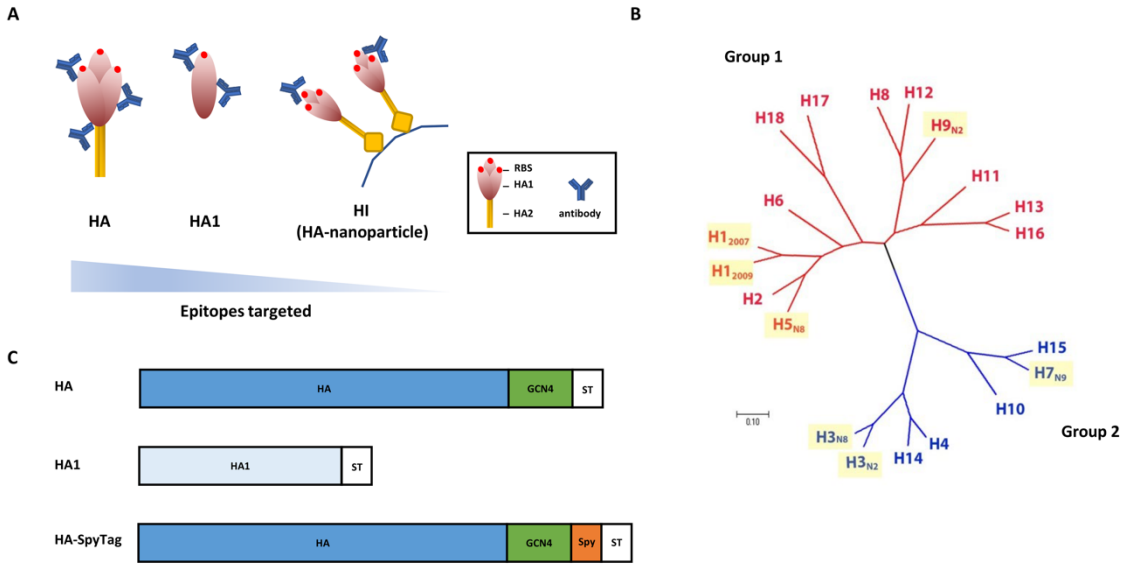

Figure 1. Recombinant soluble HA proteins used in this study. (A) Schematic depiction of antibody interaction with HA trimeric protein, HA1 protein and HI (HA-conjugated nanoparticle). The triangle indicates the different number of epitopes targeted in the different assays. (B) Phylogenetic tree indicating genetic relationship of HAs from different influenza A subtypes. HA proteins used in this study are marked in yellow. (C) Schematic representation of the recombinant HA proteins, HA1 proteins and HA-SpyTag proteins. The HA protein contains the HA1 and HA2 subunits fused to the GCN4 isoleucine zipper trimerization motif (GCN4) and Strep-tag II (ST). The HA1 protein contains the HA1 subunit of HA protein and ST. The HA-SpyTag protein has a schematic structure similar to that of the HA protein but carries an additional peptide SpyTag (Spy).

3.2 Production and functional assessment of HA proteins and HA-conjugated nanoparticles

Recombinant soluble HAs were genetically fused to a Strep-tagged GCN4 domain to ensure expression in their native trimeric form [36], while HA1s were only provided with a Strep-tag (Figure 1C). Protein expression and purification was analyzed by western blot analysis with reference antisera of anti-$H1_{2009}$, -$H3_{N2}$, -$H3_{N8}$, -$H5_{N8}$, -$H7_{N9}$ and $H9_{N2}$. All HA and HA1 proteins displayed electrophoretic mobilities

corresponding to their expected molecular weights and displayed reactivity with the corresponding reference sera (Figure S1). As expected, for some sera cross-reactivity with other HAs could also be observed, which was particularly apparent for Anti-$H3_{N8}$ with $H3_{N2}$.

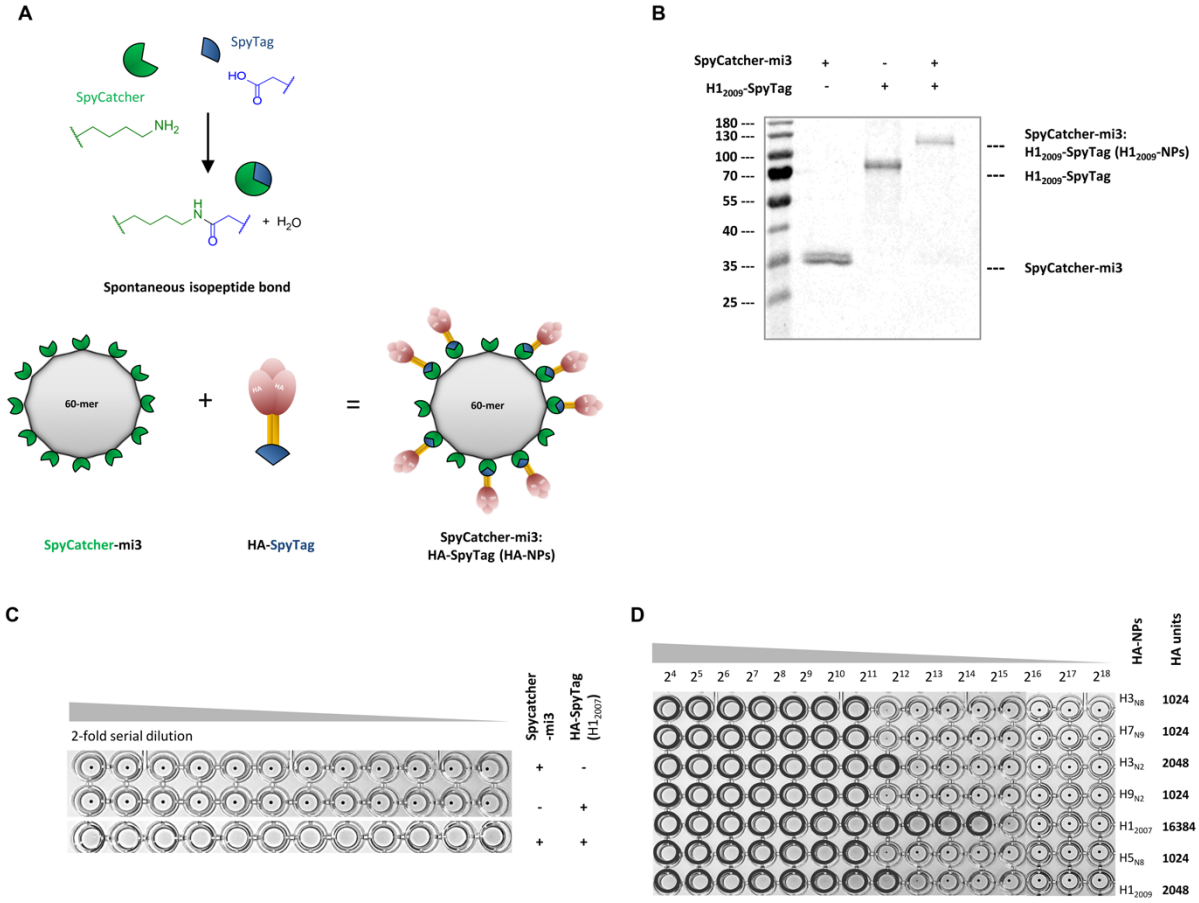

Figure 2. Nanoparticles displaying HAs have hemagglutination activity. (A) Cartoon representation of self-assembly of HA-nanoparticles (HA-NP) by forming spontaneous isopeptide bonds between SpyTag-HA protein and SpyCatcher-mi3 nanoparticles. (B) SpyCatcher-mi3 NPs were incubated with HA-SpyTag at a 1:1.5 molar ratio at 25 °C for 36 h, followed by analysis of HA-NP formation via reducing SDS-PAGE. (C) Hemagglutination by HA is dependent on its multivalent presentation on nanoparticles, as shown by a hemagglutination assay with human erythrocytes. (D) HA units obtained with the HA-NPs displaying different HAs.

In the present study, we also explored the possibility of performing HI assays without the need of virus propagation. Since the receptor-binding avidity of soluble trimeric HA is not high enough to efficiently agglutinate erythrocytes, multivalent presentation of HA trimers is needed [36]. To achieve this, we expressed our HA proteins with a C-terminal SpyTag (Figure 1C) and conjugated the trimeric HAs to a SpyCatcher protein genetically fused to self-assembling mi3 nanoparticles (NPs; Figure 2A). SpyCatcher-mi3: HA-SpyTag conjugation results in spontaneous formation of isopeptide bonds (Figure 2A, see also reference [37]). High conjugation efficiency between SpyTagged HA and SpyCatcher-mi3

was confirmed by the altered electrophoretic mobility of the conjugated proteins in polyacrylamide gels (Figure 2B).

Next, the ability of the SpyCatcher-mi3:HA-SpyTag nanoparticles (HA-NPs) to support hemagglutination was analyzed. Strong agglutination of human erythrocytes was indeed observed for NPs displaying HA (HA-NP), but not for trimeric HAs or "empty" NPs (Figure 2C). $H1_{2007}$-NPs exhibited more than 1024 hemagglutination units at 1 µM concentration. We next tested NPs decorated with each of the 7 SpyTagged HAs, resulting in hemagglutination units between 1024 and 13684 (Figure 2D). The data show that NPs decorated with trimeric HAs display high hemagglutination activity and indicate that these NPs can be used for serology screening using the HI assay.

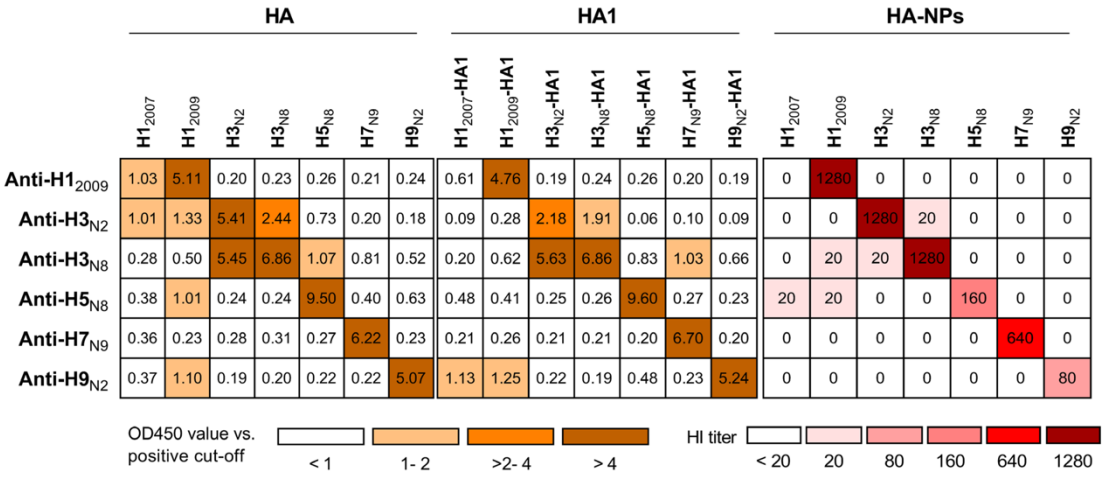

Figure 3. Reactivity of influenza HA type-specific antisera against different HAs. Reactivity was measured by HA or HA1 ELISA and by HI using HA-NPs. ELISA results are expressed as ratios of the OD_{450} value divided by the positive cut-off (five times the background). HI titers of <20 are represented as "0" in the figure.

3.3 Validation of HA ELISA, HA1 ELISA and NP-based HI assay with reference antisera

The half-maximal effective concentration (EC_{50}, expressed as dilution ratio) of each reference antiserum against the matching HA or HA1 was determined in an indirect ELISA format (Table S1). Next, all reference sera were analyzed by ELISA against all seven HA and HA1 proteins at their EC_{50}. As indicated in Figure 3, all reference antisera exhibit potent antibody response against matching HA and HA1 proteins (ratios of OD_{450} value and positive cut-off values between 2.18 and 9.60). Subtype-specific reactivity could readily be distinguished via this analysis, since the reactivity of each antiserum against HA or HA1 proteins of the corresponding subtype is at least 4-fold higher than that against the others (Figure 3). The reactivity of the anti-H1 serum was lineage specific, since little reactivity against $H1_{2007}$ was observed. In contrast, the two H3-specific sera reacted with both H3 proteins. Generally, in comparison with HA, much less cross-reactivity of the different sera was observed with the HA1

proteins. These data validate the recombinant HA and HA1 proteins as versatile tools for performing high-throughput ELISA screening of serum samples.

We also explored the inhibitory properties of the reference antisera with HI assays by using the different HA-NPs. HI titers obtained with different combinations of reference antisera and HA-NPs are summarized in Figure 3. All reference antisera display prominent HI titers (80 to 1280) against their homologous HA-NPs. Noticeably, antibody responses detected by HI is not just subtype specific but also strain specific. Thus, the two H3-specific sera only yielded high HI titers with the homologous H3-NPs (Figure 3). We conclude that the HA- and HA1-ELISAs combined with the NP-based HI assay are complementary in the detection of humoral responses against closely and distantly related IAVs.

3.4 IAVs seroprevalence in cats

Feline sera (n=122) collected from shelters in 2016 (2016 cohort) were successively screened for influenza-specific antibodies using the HA and HA1 ELISAs as well as the nanoparticle-based HI assay (Figure 4 and Table S2). In total, 29 of the 122 sera (23.6%) contained anti-HA antibodies detectable in the HA ELISA. The majority of responses are against $H1_{2009}$ (17/122, 13.9%), followed by $H5_{N8}$ (9/122, 7.3%), $H1_{2007}$ (7/122, 5.7%), $H7_{N9}$ (4/122, 3.2%), $H3_{N8}$ (3/122, 2.4%), $H3_{N2}$ (2/122, 1.6%) and $H9_{N2}$ (1/122, 0.8%) (Figure, 4A and B). Although 21 sera reacted with only one HA protein, 8 sera exhibited reactivity against more than one HA, as shown by HA ELISA (Figure 4 and Table S2, row number C to E, G to I and M). Of the eight serum samples that reacted with multiple HAs, one serum sample was positive with four HAs (row number G), four sera with three (row number D, H and I) and three (row number C, E and M) serum samples with two HAs. Most of the cross-reactivity occurred with sera that display reactivity with both $H1_{2007}$ and $H1_{2009}$ (see Table S2, row number A to J), with six out of seven sera positive for $H1_{2007}$ also being positive for $H1_{2009}$.

Antibody responses obtained with the HA1 ELISA are expected to display less cross-reactivity. Of the 29 HA positive sera, only 14 serum samples were positive in the HA1 ELISA (Figure 4C; Table S2). The pattern of positive response against HA1 is similar to HA. The majority of the sera reacted against HA1 of $H1_{2009}$ (9/122, 7.3%), followed by $H5_{N8}$ (4/122, 3.2%), $H3_{N8}$ (2/122, 1.6%) and $H1_{2007}$ (2/122, 1.6%). All HA1 positive sera are also positive for HA (Table S2). Notably, the serum samples positive for both H1 proteins, were only positive for $H1_{2009}$-HA1, in agreement with less cross-reactivity being observed in the HA1 versus the HA ELISA. The serum sample positive for $H1_{2007}$ only, also remained positive in the $H1_{2007}$-HA1 ELISA, although it was negative in the HA-NP HI assay (Table S2, row number K).

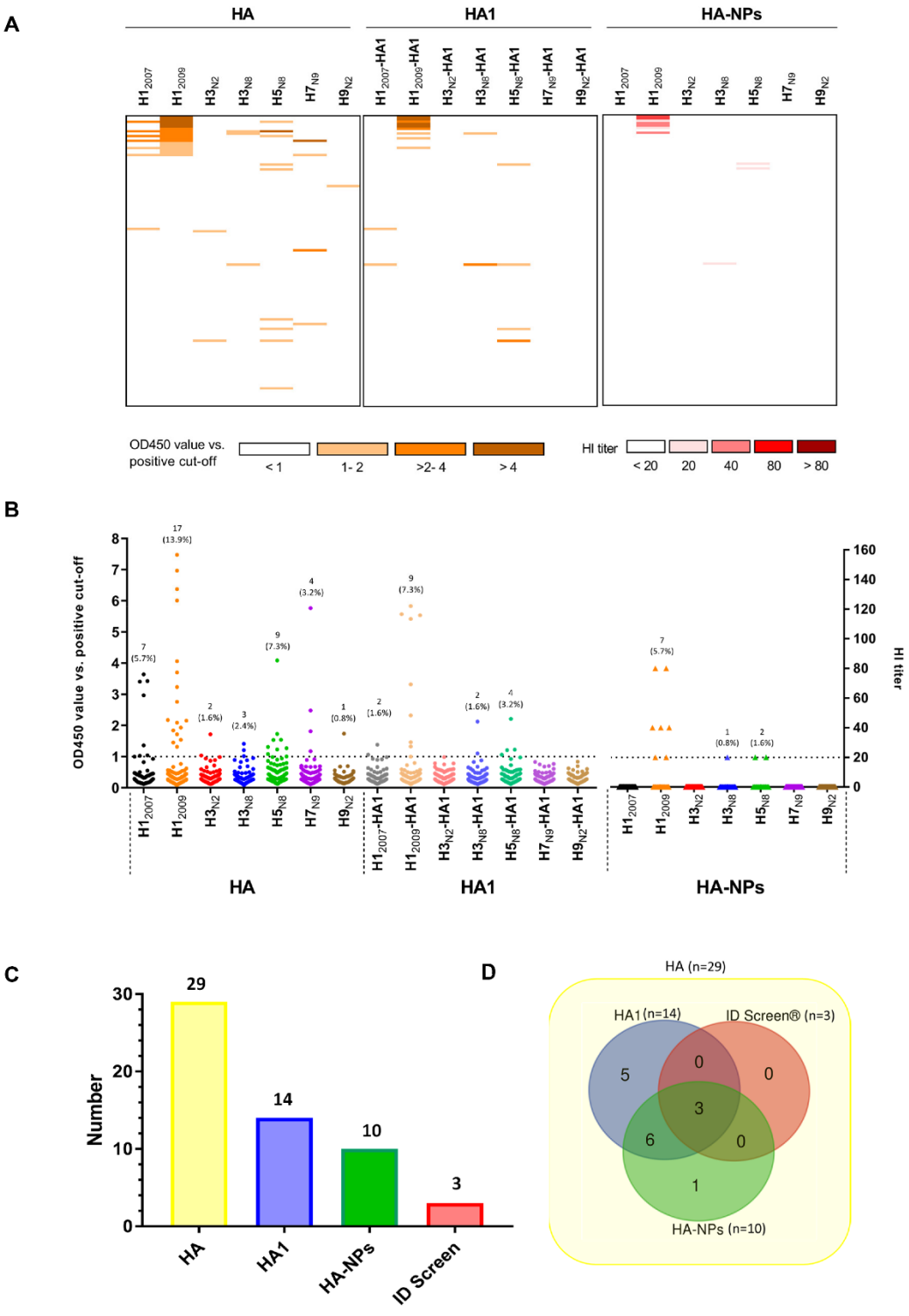

Figure 4. Seroprevalence of antibodies in shelter cats from 2016. Reactivity of shelter cat samples (2016 cohort, n=122) against different HA proteins as measured by ELISA (the two left panels) and nanoparticle-based HI assay (right panel). Reactivity profiles of all serum samples are displayed as a heatmap (A) and a distribution dot plot (B). The number and percentage of seropositive samples are indicated for each protein, and the dashed line indicates the positive threshold. Experiments were performed as in figure 3. (C and D) The number of positive samples is graphed for each assay performed (C), while their overlap is shown in a Venn diagram (D).

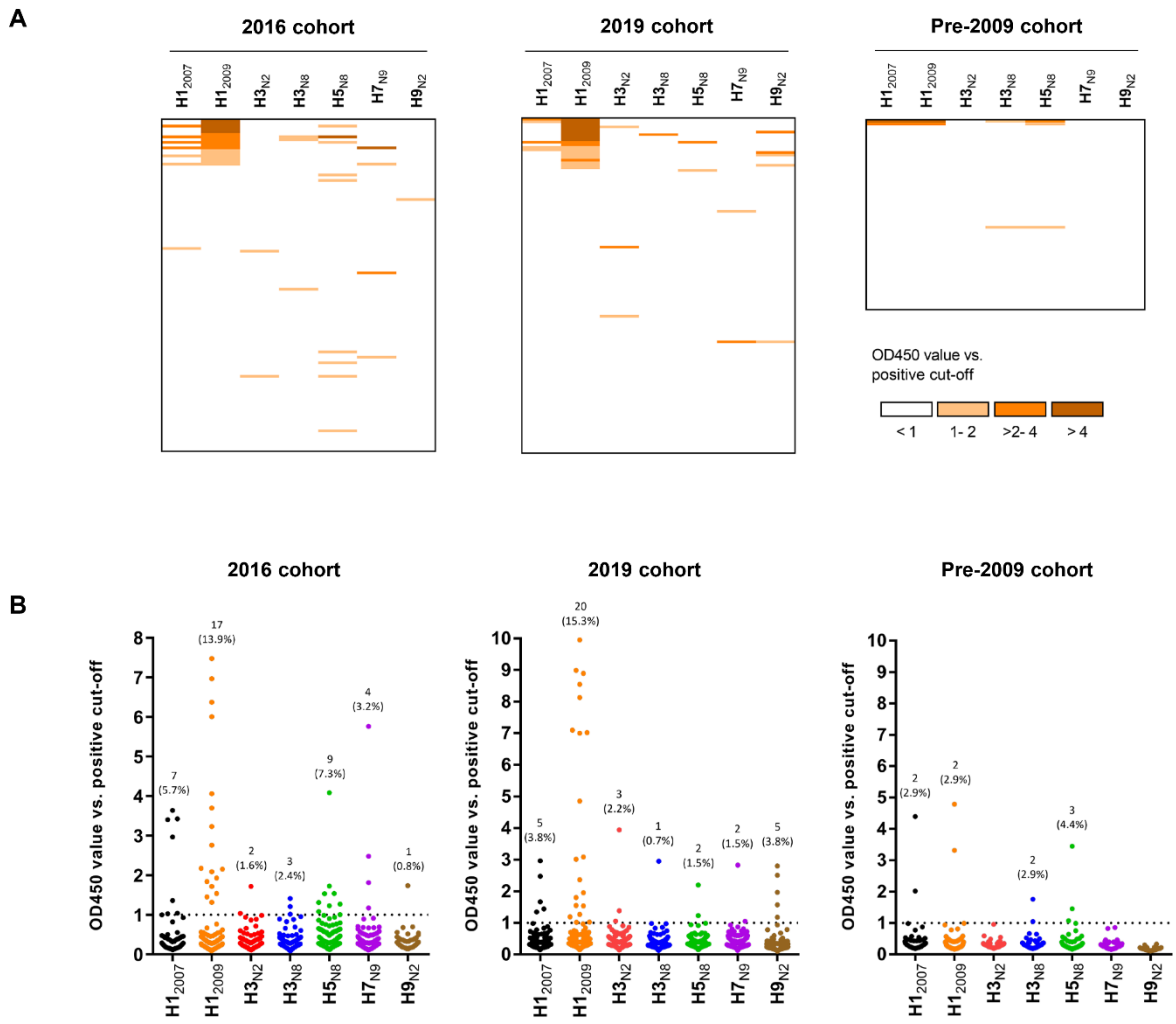

Figure 5. Seroprevalence of antibodies in cats from different cohorts. A comparison of reactivity against different HAs of samples from three cat cohorts: 2016 cohort (n=122; left panel), the 2019 cohort (n=131; middle panel), and the pre-2009 cohort (n=68; right panel) as measured via HA ELISA. The results are presented as in Figure 4.

Subsequently, all 122 cat sera were tested in nanoparticle-based HI assays. In total, 10 serum samples were able to inhibit hemagglutination, of which seven sera are positive for $H1_{2009}$-NPs, two for $H5_{N8}$-NPs and one for $H3_{N8}$-NPs (Figure 4; Table S2). Of note, all the responses observed via HI are strictly strain specific; no cross-reactivity was observed, even with the serum samples that are positive against two or more HAs as tested by ELISA. All but one samples positive in the HA-NP HI were also positive in the HA and HA1 ELISA. To further validate our observations, the seven $H1_{2009}$-NPs positive sera were analyzed side by side via HI assays with HA-NPs and H1N1 virus particles, together with five cat serum samples tested negative in this study and seven SPF cat serum samples. The data shows that the HI results obtained with NPs and virus particles are nearly identical (see Table S3). In short, the results indicate that shelter cats (2016 cohort) often contain antibodies against human and/or avian influenza

viruses, particularly against H1N1pdm09. The highest number of seropositivity was observed with the HA ELISA, while the lowest number was obtained with the more specific HI assay.

All the HA ELISA-positive cat sera from the 2016 cohort were also analyzed with a commercial competitive nucleoprotein ELISA kit (ID Screen ELISA) that was used in several cat and dog IAV serological studies [15,41]. Only 3 of the 29 HA-positive cat sera were determined to be positive in the ID Screen ELISA (Figure 4, see also Table S2), and a poor agreement between HA ELISA and ID Screen ELISA was found by Kappa statistics (kappa = 0.019). A selection of 31 cat sera that were negative in our ELISA and HI were all negative in ID screen ELISA (see Table S2, row U).

Since high IAV seroprevalence was observed in samples collected from shelter cats in 2016, we also investigated antibody responses from household owned cats. Samples were collected in 2019 or pre-2009, i.e., before the H1N1pdm09 outbreak. Comparison of ELISA reactivity against HA proteins of three cat cohorts is summarized in Figure 5. Household owned-cat samples from 2019 showed a pattern of IAV reactivity similar to that observed for shelter cats. Thus, reactivity could be observed with all HAs, with the majority of responses being against HA of $H1_{2009}$ (15.3%). As expected, the pre-2009 cohort showed a significantly lower (p= 0.0141) number of positive sera for $H1_{2009}$ compared to the post-2009 samples (Table 1). Of the three serum samples from the pre-2009 cohort that were positive for HA, two were also positive in HA1 ELISA, while none of them displayed hemagglutination inhibition (Figure S3). When combining all cat sera results obtained in the HA ELISAs, significantly (p< 0.0001) more sera were positive for $H1_{2009}$ than for $H3_{N2}$, both of which are HAs derived from human viruses (Table 1).

3.5 IAVs seroprevalence in dogs

Canine sera (n=154) collected in 2019 (2019 cohort) and prior to 2009 (pre-2009 cohort) were also screened by HA- and HA1-ELISA and NP-based HI. As summarized in Figure 6 and Table S4, 21 serum samples (13.6%) of the 2019 cohort were positive for HA, while 16 (10.3%) and 14 (9.0%) sera were positive in HA1 ELISA and HI assay, respectively. Of the HA ELISA positive sera, the majority was directed against $H1_{2009}$ (n=19), two serum samples against $H5_{N8}$, and one serum sample was positive for $H7_{N9}$. Only one serum sample reacted with more than one HA in the HA ELISA (Figure 6; see Table S4, row A). HA-ELISA-positive dog sera from 2019 cohort were also analyzed with ID Screen N protein ELISA (Figure 6, see Table S4). Four of 21 dog sera were tested positive, and a poor agreement between HA ELISA and ID Screen ELISA was found for dog sera (kappa = 0.289) similar to the results obtained with the cat sera. Fourteen ELISA- and HI-negative dog sera were all tested negative in the ID screen ELISA (see Table S4, row K). The pre-2009 significantly differed (p= 0.0038) from the 2019 cohort, since no reactivity against $H1_{2009}$ was found (Figure 6 and Table 1). Similar to

the 2019 cohort, the pre-2009 cohort contained two samples that were positive for H7 in all assays (Figure S3).

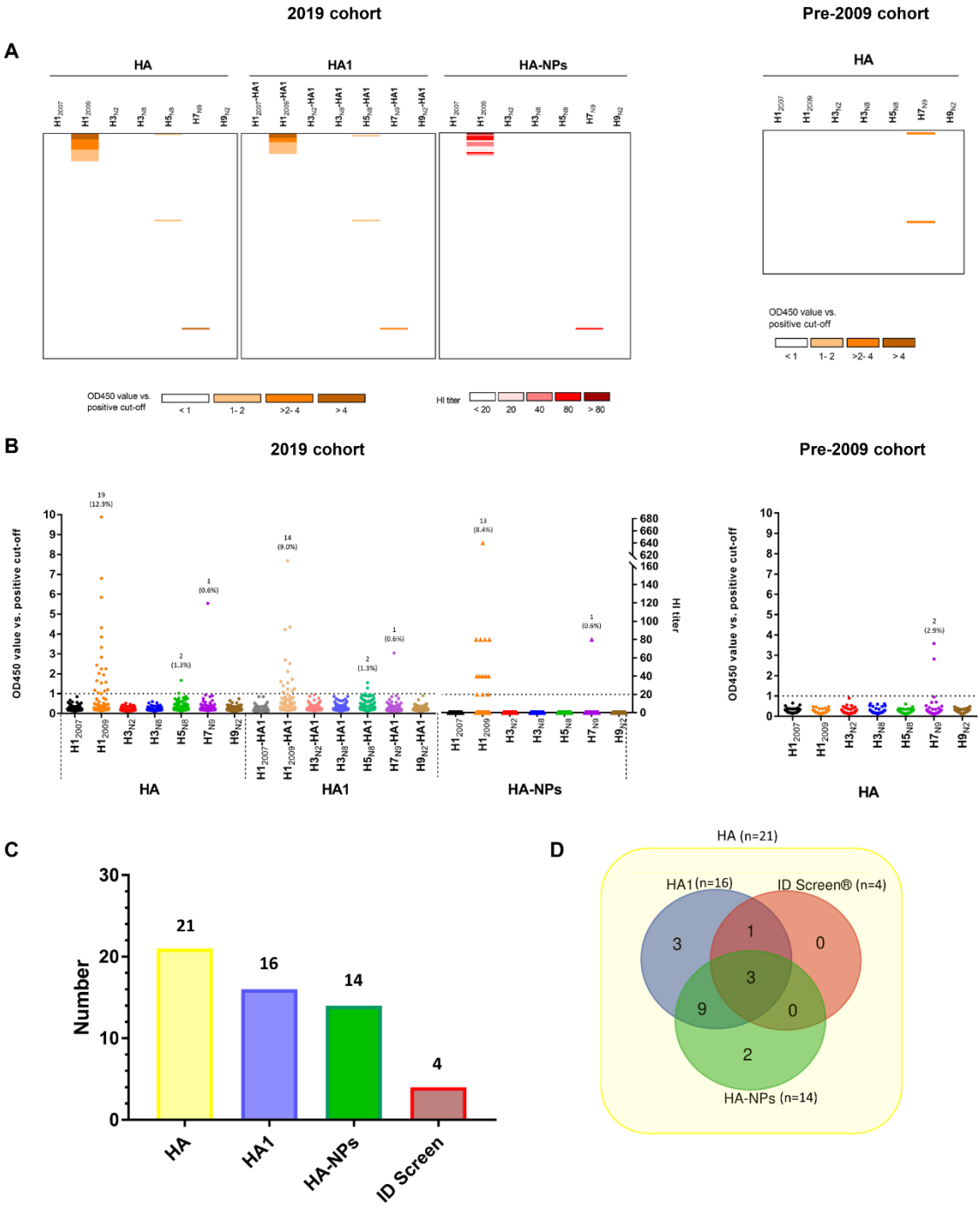

Figure 6. Reactivity of dog serum samples against different HA proteins. Reactivity profiles of all samples are displayed as a heatmap (A) and a distribution dot plot (B), similarly to that shown in Figure 4. Results for the 2019 cohort (n=154) for all three assays are shown on the left. The results for the pre-2009 cohort (n=68) for the HA ELISA are shown on the right. (C and D) The number of positive samples from

the 2019 cohort is graphed for each assay performed (C), while their overlap is shown in a Venn diagram (D).

Comparison of the results obtained with the cat and dog sera (Table 1) indicates that sera from these two species did not differ in the seroprevalence of H1 antibodies, which for both species resulted in significantly ($p< 0.0001$ in both cases) higher number of positive sera for $H1_{2009}$ than for $H3_{N2}$. Several cat sera were positive for HA, but not for HA1, of $H1_{2009}$, which was much less observed for dogs (Figure 4 and 6). As a result, analysis of the Pearson correlation between the OD values obtained with the $H1_{2009}$ HA- and HA1-ELISA (Figure S2) gave a higher value for dogs ($R^2 = 0.959$) than for cats ($R^2 = 0.812$). Furthermore, the dog and cat sera significantly differed ($p= 0.0069$) in the seroprevalence of antibodies specific for avian IAVs, which was higher in cats than in dogs.

Table 1. Summary of antibody responses against different HAs by HA ELISA in cats and dogs

			No. of samples			
Species	**Cohort**	**HA**	**Total**	**Positive**	**Prevalence[a]**	**95% CI[b]**
Human IAVs						
Cat	pre-2009	$H1_{2009}$	68	2	0.029	0.002-0.107
	2016 and 2019	$H1_{2009}$	253	37	0.146	0.108-0.195
	pre-2009, 2016 and 2019	$H3_{N2}$	321	5	0.016	0.006-0.037
Dog	pre-2009	$H1_{2009}$	68	0	0.000	0.000-0.064
	2019	$H1_{2009}$	154	19	0.123	0.080-0.185
	pre-2009 and 2019	$H3_{N2}$	222	0	0.000	0.000-0.021
Avian IAVs						
Cat	pre-2009, 2016 and 2019	$H5_{N8}$, $H7_{N9}$ and $H9_{N2}$	321	25	0.078	0.053-0.113
Dog	pre-2009 and 2019	$H5_{N8}$, $H7_{N9}$ and $H9_{N2}$	222	5	0.023	0.008-0.053

[a]Prevalence = positive number/sample number.
[b]95%CI, 95% confidence interval (computed by the modified Wald method)

4 Discussion

To date, several serological studies of IAV in cats and dogs have been performed, and seroprevalence of antibodies against several different IAV subtypes has been observed [reviewed in reference [14]]. Most of these studies are based on samples collected from animals in Asia and the United States. As a consequence, information regarding the circulation of IAV in cat and dog population in Europe is still limited. In the present study, we analyzed antibody responses in sera collected from different Dutch cat and dog cohorts against HA proteins of different IAV subtypes by applying a combination of newly developed serological assays that differ in their specificity and sensitivity. By using these different assays, we demonstrate a high seroprevalence of IAV-specific antibodies in sera from both species, particularly against the H1N1pdm09 virus. Several cats and dogs also displayed reactivity against avian IAVs, thereby indicating the potential of these animals to serve as an IAV mixing vessel.

IAV HA contains an immunodominant, but divergent, HA1 subunit and an immunosubdominant, but highly conserved HA2 subunit. The HA1 subunit is commonly used as antigen in a protein microarray format for assessing vaccination efficacy [42,43]. Recently, this method was also used for IAV

seroprevalence studies in chicken or horse populations [44,45]. However, by only focusing on HA1, antibodies against more divergent strains might be missed. Therefore, in the present study, in addition to HA1, we also used complete HA ectodomains stabilized in their native trimeric conformation in ELISAs. The ELISA with trimeric HA allows detection of antibodies against epitopes present in both the HA1 and HA2 subunit, which maximizes detection of IAV antibodies, including potential cross-reactive antibodies elicited by more distantly related HAs that fail to recognize HA1 but still target the conserved HA2 subunit. These ELISAs were complemented with highly specific HI assays, which specifically detect antibodies targeting epitopes surrounding the RBS. Combining complementary assays differing in specificity and sensitivity allowed us to observe a high seroprevalence of IAV-specific antibodies in cats and dogs, which for several animals could be confirmed by highly specific HI assays. While the assays developed within this study are not likely to provide help with clinical decision making, they will be of use for research and public health investigations. Of note, an often-used commercially available ELISA, in which the highly conserved nucleoprotein is used as antigen, suffered from very low sensitivity compared to our in-house HA and HA1 ELISAs, which partly may explain the low seroprevalence normally observed in studies using similar nucleoprotein-based ELISAs [15,41].

By multivalently presenting HA on nanoparticles we developed a reliable HI assay. Traditionally, HI is performed with live or inactive virus particles, for which virus propagation is necessary. Obviously, this cannot be easily applied in all laboratories, particularly when Biosafety Level 3 containment is required. By using recombinant protein-based assays, this issue may be resolved, resulting in HI assay platforms that are easy to perform and standardize. Recently, a similar HA- NP design was used for immunization studies, and effective presentation of HA proteins was confirmed by electron microscopy [46]. A comparable approach was also conducted for Middle East respiratory syndrome coronavirus (MERS-CoV) serology, where the sialic acid binding MERS-CoV spike $S1^A$ domain was presented on NPs to perform HI assay [47]. In the present study, we showed that results obtained with HA-NPs and virus particles are nearly identical (Table S3), indicating that the HI assay with HA-NPs may be used as an alternative for the HI assay with virus particles, which is currently regarded as the gold standard [48].

We analyzed the prevalence of IAV-specific antibodies in cat and dog samples from different cohorts. In the 2016 and 2019 cat cohorts, positive reactivity was noticed with HA of each of the seven IAV strains via HA ELISA, with most responses against $H1_{2009}$ (Figure 4 and 5). Similarly, the majority of seropositivity in the 2019 dog cohort was observed for $H1_{2009}$. Both for cats and dogs, H1 reactivity was significantly higher (p_{cat}= 0.0141, p_{dog}= 0.0038) in samples collected after 2009 than in samples collected prior to 2009, whereas reactivities against other subtypes were not significantly higher (see Table S5 and S6). The H1 reactivity in post-2009 cohorts is also significantly higher (p< 0.0001 in both

cases) than the H3 reactivity (Table 1). This might be explained by H1N1pdm09 being more capable of crossing the host species barrier than the human H1N1 virus that circulated prior to 2009 or the human H3N2 virus. While the reason for this difference is not known, it may be related in part to differences in the receptor-binding properties of these different human viruses, with H1N1pdm09 being able to bind to α2,3-linked, in addition to α2,6-linked, sialic acid receptors, which contrasts with pre-2009 H1N1 that poorly binds α2,3-sialic acid [49].

We provide the first serological evidence of infection of cats with H3, H5, H7 and H9 subtypes in Europe. Such observations are in agreement with seroprevalence studies of cat sera conducted in Asia and the United States [41,50,51]. Although a significantly lower (p= 0.0069) seroprevalence of antibodies against avian IAVs could be detected in dogs compared to cats (Table 1), in some dog sera antibody responses against H5 and H7 were detected using different assays, including HI for H7. This is the first evidence of infection with H5 and H7 subtype viruses in European dogs. The seropositivity of cat and dogs is likely due to spillover of avian IAVs from birds to cats and dogs. Apparently, this happens more often for cats than for dogs, in agreement with their predatory behavior. Interestingly, both the H5-and H7-positive sera are from hunting dogs that might chase birds. The presence of H7-specific antibodies in dogs has so far only been reported once for one dog in Africa [52].

In conclusion, our findings demonstrate the value of using comprehensive serological assays to analyze antibodies against IAVs and for improved serosurveillance of IAV infections. Although we only analyzed cat and dog sera, it will be of interest to analyze to what extent these assays will be applicable for serosurveillance of (zoonotic) IAV infections in humans, which is currently particularly being done using HA1-based assays [53]. Our results indicate that infection of cats and dogs with several subtypes of IAVs is prevalent. This emphasizes the potential role of both animal species as mixing vessels for IAVs, with the possibility of the emergence of mutated/reassorted viruses with increased zoonotic potential. Recurrent epidemiological surveillance for influenza infections among cats and dogs is needed, which could serve as an early cautioning system for human and animal threats.

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Supplementary information

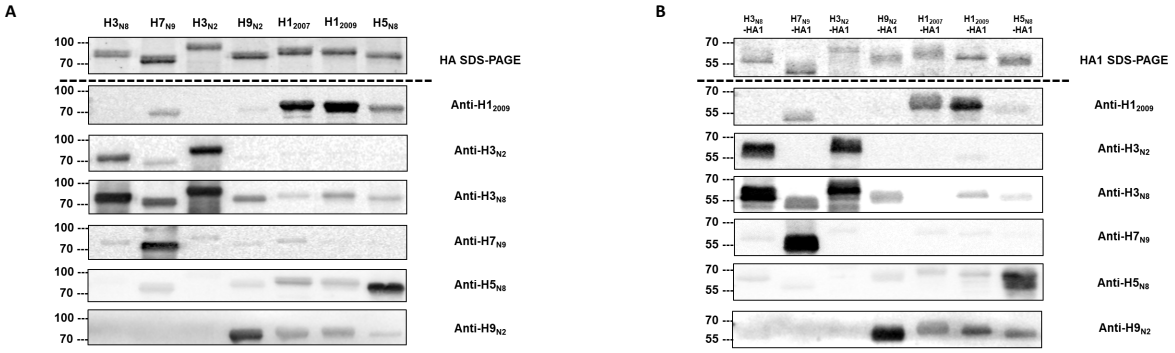


Figure S1. Reactivities of reference antisera against different HA (A) and HA1 (B) proteins analyzed by western blot analysis.

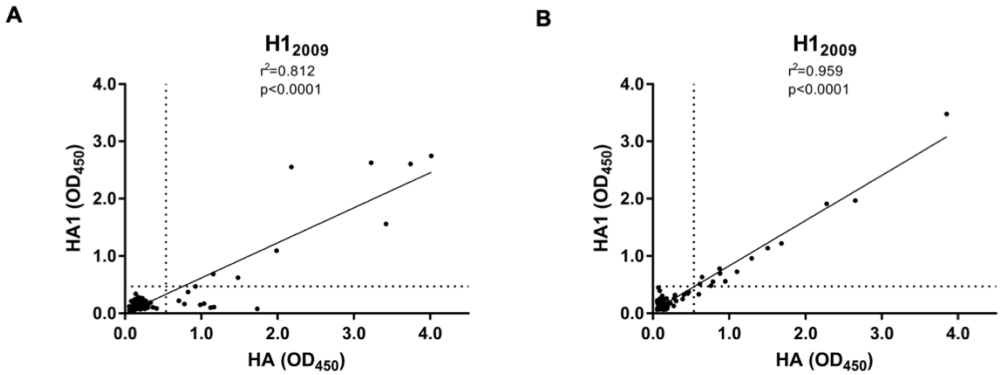


Figure S2. Correlation between the OD values obtained with HA and HA1 ELISA of H12009. (A) Cat serum samples from 2016 cohort; (B) dog serum samples from 2019 cohort. Correlation and regression coefficients were shown above the figures.

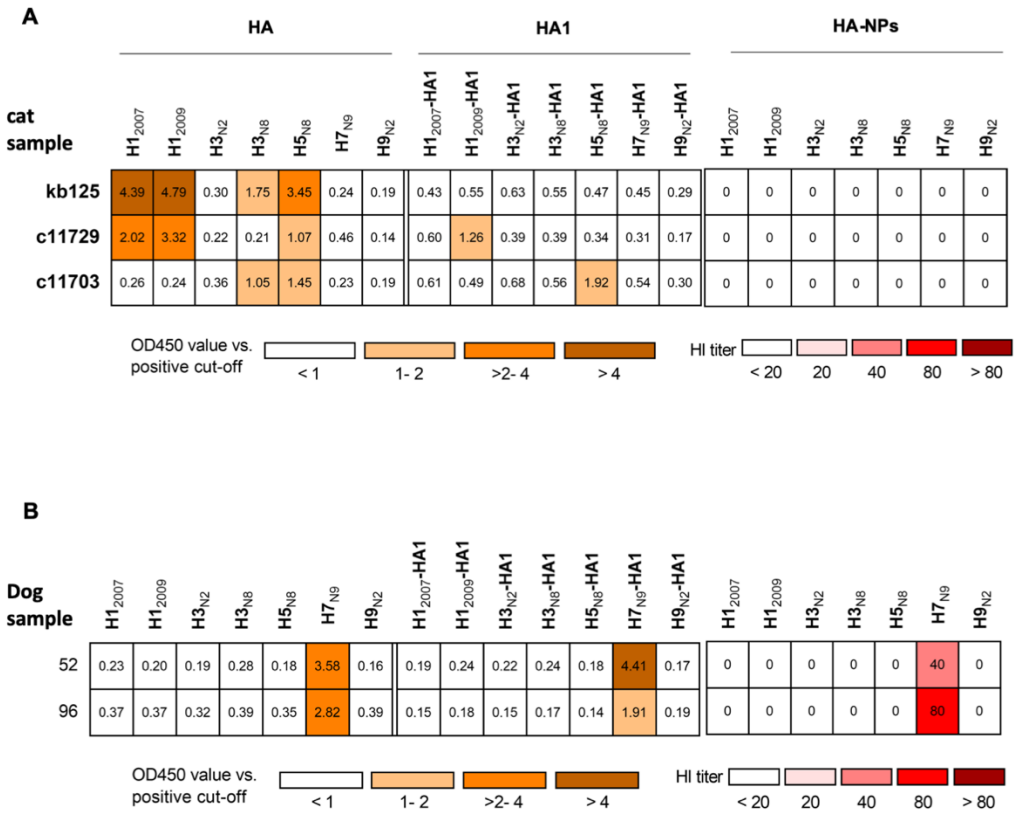


Figure S3. Reactivity of cat (A) and dog (B) serum samples collected before 2009 against different HAs as measured by HA ELISA, HA1 ELISA and nanoparticle-based HI. ELISA results are expressed as ratios of OD_{450} value divided by the positive cut-off (OD_{450} value vs positive cut-off).

Table S1. Half maximal effective concentration (EC_{50}) determined of each reference serum against corresponding HA and HA1 proteins. Results are expressed as dilution ratios.

	anti-$H1_{2009}$	anti-$H3_{N2}$	anti-$H3_{N8}$	anti-$H5_{N8}$	anti-$H7_{N9}$	anti-$H9_{N2}$
HA	1:1416	1:232	1:2847	1:2710	1:14371	1:182
HA1	1:838	1:41	1:2547	1:2632	1:15404	1:70

Table S2. Numbers of all HA positive (n=29) and 31 negative cat samples from 2016 cohort with different combinations of reactivity observed. Positive reactions are colored in orange.

	HA-ELISA							HA1-ELISA							HA-NPs							ID Screen®	Number of cats (Total=29)
Row nr.	$H1_{2007}$	$H1_{2009}$	$H3_{N2}$	$H3_{N8}$	$H5_{N8}$	$H7_{N9}$	$H9_{N2}$	$H1_{2007}$	$H1_{2009}$	$H3_{N2}$	$H3_{N8}$	$H5_{N8}$	$H7_{N9}$	$H9_{N2}$	$H1_{2007}$	$H1_{2009}$	$H3_{N2}$	$H3_{N8}$	$H5_{N8}$	$H7_{N9}$	$H9_{N2}$		
A		■							■							■						■	3
B		■							■							■							2
C		■		■					■		■					■							1
D	■	■			■				■							■							1
E	■	■							■														1
F		■							■														1
G	■	■		■	■																		1
H	■	■			■																		1
I	■	■				■																	2
J		■																					4
K	■							■															1
L			■																				1
M			■		■							■											1
N				■				■			■	■						■					1
O					■							■							■				1
P					■							■											1
Q					■														■				1
R					■																		2
S						■																	2
T							■																1
U																							31

Table S3. Comparison of hemagglutination inhibition (HI) titers of cat serum samples against $H1_{2009}$-NPs and H1N1 virus particles. Negative sera (HI titer <20), are represented as 0 in the table.

Characteristic	number	$H1_{2009}$-NPs	H1N1
Shelter cat serum samples (2016 cohort)	UFB 33	80	80
	UFB 93	80	80
	UFB 22	20	20
	UFB 74	40	40
	UFB 277	40	20
	UFB 279	20	20
	UFB 31	40	40
	UFB 11	0	0
	UFB 17	0	0
	UFB 18	0	0
	UFB 14	0	0
	UFB 23	0	0
FCoV specific sera derived from SPF cats	cat 14	0	0
	cat 15	0	0
	cat 91	0	0
	cat 93	0	0
	cat 95	0	0
	cat 115	0	0
	cat 131	0	0

Abbreviations: H1N1, Influenza virus A/Netherlands/602/2009; $H1_{2009}$-NPs, SpyCatcher-mi3: $H1_{2009}$-SpyTag nanoparticles; FCoV, feline coronavirus; SPF, specific pathogen free

Table S4. Numbers of all HA positive (n=21) and 14 negative dog samples from 2019 cohort and different combinations of reactivity observed. Positive reactions are colored in orange.

	HA-ELISA							HA1-ELISA							HA-NPs							ID Screen®	Number of dogs (Total=21)
Row nr.	$H1_{2007}$	$H1_{2009}$	$H3_{N2}$	$H3_{N8}$	$H5_{N8}$	$H7_{N9}$	$H9_{N2}$	$H1_{2007}$	$H1_{2009}$	$H3_{N2}$	$H3_{N8}$	$H5_{N8}$	$H7_{N9}$	$H9_{N2}$	$H1_{2007}$	$H1_{2009}$	$H3_{N2}$	$H3_{N8}$	$H5_{N8}$	$H7_{N9}$	$H9_{N2}$		
A																							1
B																							1
C																							2
D																							7
E																							2
F																							1
G																							2
H																							3
I																							1
J																							1
K																							14

Table S5. Summary of antibody responses against each HA by HA ELISA in pre-2009 or post-2009 cat cohorts.

	Pre-2009				Post-2009				Pre-2009 vs. Post-2009
HA	**Sample number**	**Positive number**	**Prevalence[a]**	**95% CI[b]**	**Sample number**	**Positive number**	**Prevalence[a]**	**95% CI[b]**	***p* value[c]**
$H1_{2007}$	68	2	0.029	0.002-0.107	253	12	0.047	0.027-0.082	0.5275
$H1_{2009}$	**68**	**2**	**0.029**	**0.002-0.107**	**253**	**37**	**0.146**	**0.108-0.195**	**0.0141**
$H3_{N2}$	68	0	0	0.000-0.064	253	5	0.020	0.007-0.047	0.2464
$H3_{N8}$	68	2	0.029	0.002-0.107	253	4	0.016	0.005-0.041	0.4664
$H5_{N8}$	68	3	0.044	0.010-0.127	253	11	0.043	0.024-0.077	0.9823
$H7_{N9}$	68	0	0	0.000-0.064	253	6	0.024	0.010-0.052	0.2041
$H9_{N2}$	68	0	0	0.000-0.064	253	6	0.024	0.010-0.052	0.2041

[a]prevalence =positive number/sample number.
[b]95%CI, 95% confidence interval (computed by the modified Wald method)
[c]*p* value, represent the significance of the difference of the prevalence between two cohorts (Chi-square test)

Table S6. Summary of antibody responses against each HA by HA ELISA in pre-2009 or post-2009 dog cohorts.

	Pre-2009				Post-2009				Pre-2009 vs. Post-2009
HA	**Sample number**	**Positive number**	**Prevalence[a]**	**95% CI[b]**	**Sample number**	**Positive number**	**Prevalence[a]**	**95% CI[b]**	***p* value[c]**
$H1_{2007}$	68	0	0	0.000-0.064	154	0	0	0.000-0.029	n/a
$H1_{2009}$	**68**	**0**	**0**	**0.000-0.064**	**154**	**19**	**0.123**	**0.080-0.185**	**0.0038**
$H3_{N2}$	68	0	0	0.000-0.064	154	0	0	0.000-0.029	n/a
$H3_{N8}$	68	0	0	0.000-0.064	154	0	0	0.000-0.029	n/a
$H5_{N8}$	68	0	0	0.000-0.064	154	2	0.013	0.001-0.049	0.3471
$H7_{N9}$	68	2	0.029	0.002-0.107	154	1	0.007	<0.0001-0.040	0.1758
$H9_{N2}$	68	0	0	0.000-0.064	154	0	0	0.000-0.029	n/a

[a]prevalence =positive number/sample number.
[b]95%CI, 95% confidence interval (computed by the modified Wald method)
[c]*p* value, represent the significance of the difference of the prevalence between two cohorts (Chi-square test)

Chapter 6

Summary and General Discussion

Coronaviruses (CoVs) and influenza A viruses (IAVs) are endemic in both human and animal populations. Their tendency to cross species barriers allows them to circulate continuously and colonize a wide range of avian and mammalian host species, including humans. Just over a century ago, the 1918 influenza pandemic caused by an avian origin H1N1 IAV affected one-third of the world's population and led to over 50 million deaths [1–3]. A few decades later in years 1957, 1968 and 2009, three other major IAV pandemics occurred, each of which resulted in severe consequences for global economy and public health [3–6]. In the meantime, apart from the established human coronaviruses (HCoVs), CoV outbreaks in humans occurred three times in the 21st century alone: the severe acute respiratory syndrome (SARS) CoV outbreak in 2002/2003, the Middle East respiratory syndrome (MERS) CoV emergence in 2012 and the currently ongoing SARS-CoV-2 pandemic [7–9]. At the time of writing, it still remains unclear whether SARS-CoV-2 will become endemic [10,11]. Companion animals are also susceptible to CoVs and IAVs, and reverse zoonosis of human CoVs and IAVs has been observed in these animals [12–14]. Such facts emphasize the role of companion animals as potential virus mixing vessels, with the possibility of the emergence of recombinant viruses with increased threats for both animal and humans. Consequently, it is of importance to study CoV and IAV infection in companion animals, and recurrent surveillance studies are necessary.

Being classically defined as the studies of proteins found in blood and secretions, serology was commonly used to study the prevalence of pathogen-specific antibodies [15,16]. Compared with molecular diagnostic methods at the acute stage of infection, serological methods have the advantage of being able to trace the infection after a longer period, as antibodies induced by viruses have long duration of persistence in blood after infection [17]. Therefore, serological assays are crucial tools that can be applied to support clinical diagnosis of viral infection, monitoring of vaccine compliance, assessments of herd immunity and seroprevalence studies in different populations. Different platforms are used for serology, including binding assays such as enzyme-linked immunosorbent assays (ELISA), lateral flow assays, immunofluorescence assays (IFA) and Western blot-based assays making use of infected cells, inactivated viruses or recombinant antigens [16]. Furthermore, functional assays that target virus infection (neutralization assay), block *in vitro* virus-receptor interaction (i.e. hemagglutination inhibition assay, HI) or enzyme inhibition assays (such as neuraminidase blocking assays) can also shed light on antibody-mediated immune responses [18–21]. In the context of this thesis, we developed and validated a set of tailor-made serological assays for specific detection of CoV and IAV antibodies in companion animals, and we assessed CoV and IAV seroprevalence in European pets.

Viral attachment proteins: better antigens for serological studies

The CoV spike (S) and IAV hemagglutinin (HA) proteins are the main viral envelope proteins that mediate virus binding to cell surface receptors. They are also type I fusion proteins which mediate

fusion of viral and cellular membrane. These two interdependent functions are carried out by different subunits of the protein, i.e. S1 and S2 for S, and HA1 and HA2 for HA. The fact that these proteins are exposed on the virion surface and execute essential function in virus entry make them the main targets of neutralizing antibodies [22,23]. As the consequence of genetic recombination and antigenic drift, S and HA carry the highest genetic variabilities across their entire viral genomes [9,24]. Therefore, S and HA are well suited as antigens that allow serological screening for CoV and IAV type specific antibodies. With the advance of molecular virology, high quality recombinant glycoprotein antigens from these viruses can be produced that antigenically mimic the glycoprotein structures on the virion surface.

In **Chapter 2**, we performed screening of 137 cat serum samples for CoV antibody presence with a library of S1 proteins from 12 different CoVs. We showed that a large percentage (56.7%) of samples contain CoV antibodies, with the majority of seropositivity (54.7%) observed for feline coronavirus (FCoV). The high seroprevalence of FCoV antibodies in Dutch cats was later confirmed independently with samples from a different cohort, as described in **Chapter 4**. Besides FCoV, several serum samples were also reactive against S1 proteins of other CoVs, including human pathogen HCoV-229E and porcine pathogens porcine epidemic diarrhea virus (PEDV) and porcine deltacoronavirus (PDCoV). Intriguingly, in our serosurvey we also showed that several cat serum samples displayed unique ELISA-reactivity against S1 of non-feline CoVs, namely HCoV-229E and PDCoV. We provided the first serological evidence of the potential role of cats as incidental hosts of other CoVs than FCoV, which supports the need of thorough study of naturally infected viruses in cats.

Similar to our studies with feline sera, in **Chapter 3** we employed equine coronavirus (ECoV) S1 protein as antigen and established an indirect ELISA method to detect ECoV antibody levels in equine serum samples in two formats: the conventional wet ELISA format (wELISA) for general laboratory usage and the dry standardized ELISA format (dELISA) that has potential of commercial kit development. Results from both ELISA formats correlated nicely and were shown to have high accuracy when compared with virus neutralization (VN) assays. Applying these methods, we were able to analyze the prevalence of ECoV antibodies in paired sera collected during an ECoV outbreak. Seroconversion in a large percentage of sample pairs could be detected with wELISA, and results from ELISA and VN showed a 100% correlation (rate). We further screened serum samples collected from different horse cohorts with unknown ECoV exposure using dELISA for antibodies against ECoV S1. The overall seroprevalence of ECoV antibodies in Dutch horse cohorts is compatible with results of serological studies of horses in Saudi Arabia [25]. In addition, our study showed that the seroprevalence in young horses is much lower than that of adult horses, indicating that the risk of ECoV infection increases with age. We also demonstrated the first evidence of the existence of ECoV infection in

Iceland, of which the horse population has been geographically isolated for over 1000 years and free from common equine infectious diseases [26].

Noticeably, ECoV expresses another surface protein besides S, namely the hemagglutinin-esterase (HE) protein, which was shown to also interact with cellular receptors [27]. Although the role of HE in ECoV infectious cycle remains to be elaborated, recent reports have shown that HE is essential to establish infection with HCoV-OC43 and bovine coronavirus (BCoV), both of which belong to subgenus *Embecovirus* and have a close genetic relationship with ECoV [28–30]. Therefore, it is reasonable to hypothesize that HE also plays a critical role in ECoV infection, and consequently might be a target for (neutralizing) antibodies. In future serological studies, HE might also be considered as an appropriate antigen candidate.

Several months after the SARS-CoV-2 outbreak, sporadic infections of SARS-CoV-2 in companion animals were reported worldwide [31–35]. Building upon previous serological studies described in this thesis, we developed and validated a set of SARS-CoV-2 serological assays for cats and dogs, and conducted the first SARS-CoV-2 seroprevalence study in companion animals in the Netherlands (**Chapter 4)**. For our ELISA-based analysis, we utilized SARS-CoV-2 S1 and the receptor-binding domain (RBD) as antigens. The SARS-CoV-2 nucleocapsid (N) protein obtained from a commercial source was also examined as an antigen since it is used in several serological studies of human serum samples. When testing samples collected in 2019 (pre COVID-19 cohort), no antibody reactivity was found against SARS-CoV-2 S1 or RBD, although high seroprevalence was noticed in those samples for endemic CoVs. However, nearly one-fifth of the pre COVID cat sera and one dog serum sample tested positive with SARS-CoV-2 N protein. Further validation with samples from FCoV infected specific pathogen free (SPF) cats showed that SARS-CoV-2 N cross-reactive antibodies are induced upon FCoV infection. All three proteins reacted with serum samples from the COVID-19 exposed cohort, but only reactivities detected with SARS-CoV-2 S1 and RBD correlated well with VN data determined with vesicular stomatitis virus (VSV) pseudotyped with SARS-CoV-2 S protein (SARS2-VSV). These observations support the usage of chimeric S protein and certainly, disqualify the N protein as antigen for serological studies of cats and dogs.

Domestic cats and dogs are progressively being recognized as IAV hosts [13,14]. In **Chapter 5**, we switched gears from CoV and investigated IAV infection in cats and dogs. We used the HA ectodomain as antigens in our ELISA and newly developed nanoparticle-based, virus-free heamagglutination inhibition (HI) assay. The HA1 subunit, which is the equivalent of CoV S1, was also expressed and used as an antigen. Experiments with reference antisera from immunized animals confirmed the antigenicity of these antigens and the viability of the assays. Notably, an often-used commercially available ELISA, in which the highly conserved IAV N protein is used as antigen, showed very low sensitivity compared to our in-house HA and HA1 ELISAs. This may partly explain the low

seroprevalence rate normally observed in studies using similar N protein-based ELISAs [36,37]. The low sensitivity may result in false negative result, and lead to false conclusions regarding IAV seroprevalence. Collectively, our research added to the importance of using HA proteins as probes for serological studies [38–42].

Notably, although S is larger than HA (monomer size 180kDa vs. 100kDa), the field of structural analysis of CoV S has advanced greatly since 2016 when the first structure of a CoV S trimer was reported [43,44]. Up to the present time, structures are available of S of representative strains from every genus of subfamily *Orthocoronavirinae* (**Chapter 1** and references therein). Studies have focused on the production of stable CoV S trimer for structural and immunization studies, likewise with the continuous optimization of the envelope glycoprotein trimer of human immunodeficiency virus for vaccine development [45,46]. Therefore, full-length CoV S trimers appeared as suitable antigens for serological studies. Employing the complete S trimer allows detection of antibodies against most S epitopes, including epitopes present on the S2 subunit, and putative conformational epitopes that do not form in monomeric/dimeric S proteins. At the time of writing, native S trimers had already been used to evaluate antibody responses post SARS-CoV-2 infection in animal models and to assess SARS-CoV-2 seroprevalence in the human population [47,48]. For future studies, it will be of interest to involve native S trimers of different CoVs as effective tools for serology of different hosts. On the other hand, gaining sensitivity by using S trimers as antigens comes with an inevitable cost of specificity. Compared with the S1 subunit, the S2 subunit is more conserved and has a higher chance of identifying cross-reactive antibodies. To this end, it is of importance to distinguish antibody responses by using different serological assays and antigens.

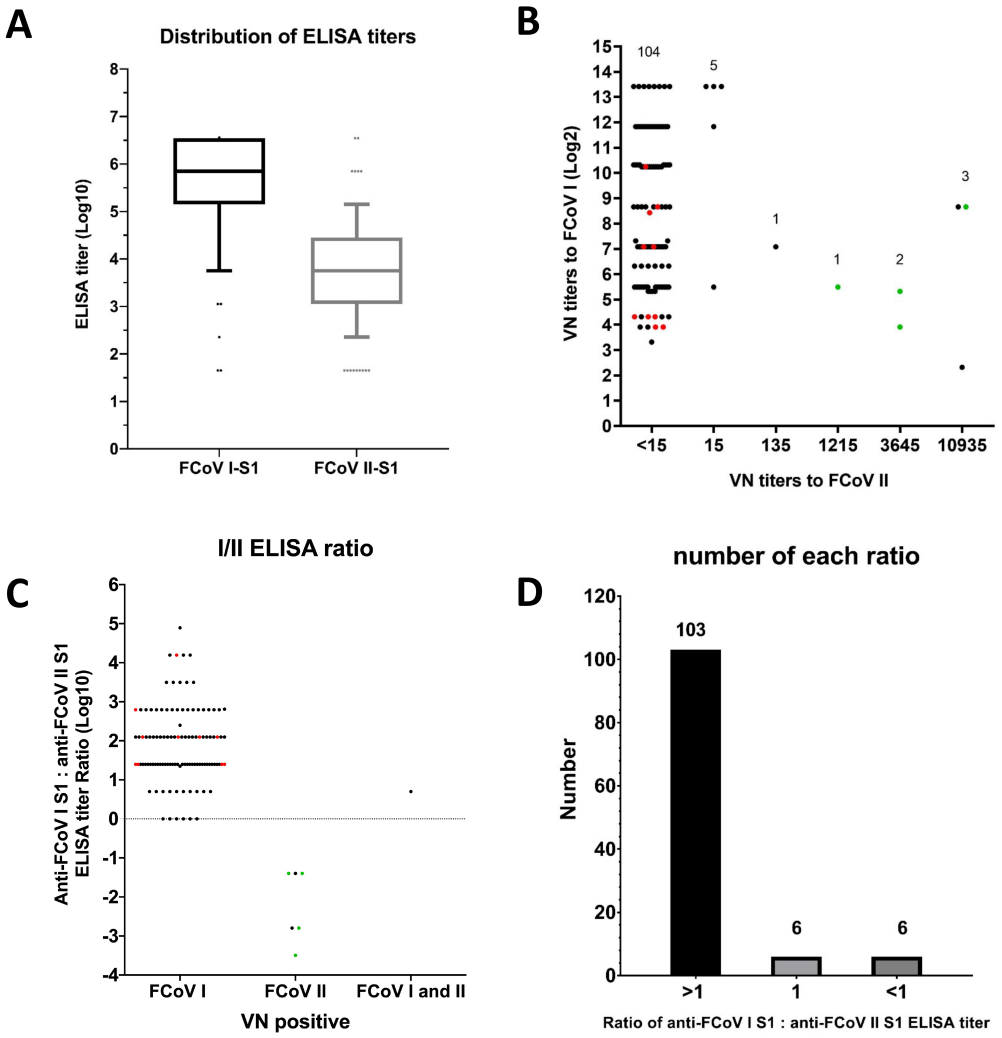
Serological differentiation of viral infections

In **Chapter 2**, we showed that many cat sera exhibit seropositivity against more than one CoV S1, especially many FCoV S1 positive samples are positive for PEDV S1-reactive antibodies. Compared to other viral proteins, the sequence identity of the S1 proteins is the lowest between related viruses, nevertheless antibody cross-reactivity against S1 antigens likely occurs. Based on our previous understanding of PEDV S1 antigenicity, we employed different PEDV S1 domains to test against sera from FCoV serotype I infected SPF cats. The data show clearly that PEDV S1 reactivity is prompted by FCoV infection, however only when the most conserved $S1^{CD}$ domain (51.2% identity between FCoV and PEDV; see **Chapter 2**) is present. The other three domains, namely $S1^{0}$, $S1^{A}$ and $S1^{B}$, do not seem to contribute to the observed cross-reactivity. Notably, the fact that anti-FCoV S1 antibodies can react with PEDV S1 does not exclude the possibility that field cats might be naturally infected with PEDV or PEDV-like viruses, hereafter being seropositive for PEDV antigens. In future studies, selective usage of different PEDV S1 domains could support discrimination of infections of FCoV and PEDV/PEDV-like viruses.

Meanwhile, the majority of cross-reactivity occurs between S1 of two FCoV serotypes, as all the FCoV serotype II S1 positive samples are also positive for FCoV serotype I S1 (**Chapter 2**). As prevalence of serotype II FCoV is low in Europe [49–52], it is possible that most of the serotype II seropositivity is driven by cross-reaction. At the time, little is known regarding the structure of FCoV S, and $S1^{0}$-$S1^{CD}$ domains are likely to be present in both FCoV serotype I and II S1 based on sequence analysis. To explore this further, we delineated the FCoV serotype I and serotype II S1 domain borders based on structural models generated with homology modeling using the cryoelectron microscopy (cryo-EM) spike protein structure of the related HCoV-NL63 as the template model [53]. All domains were successfully expressed, and their antigenicity was assessed with cat serum samples (**Chapter 2**). After we finished our studies, Yang et al. reported the first structure of FCoV S, and different S1 domains were structurally identified [54]. Pairwise alignment shows that the domains predicted by us are highly comparable to the ones defined by cryo-EM. Similar to FCoV and PEDV, the $S1^{CD}$ domain induces the largest part of cross-reactivity between FCoV serotype I and II, as shown by ELISA with specific antisera from experimentally infected SPF cats.

While FCoV serotype I-specific sera could react with serotype II S1, the apparent ELISA-reactivity was not as high as with serotype I S1. Such observations led us to explore whether the relative ELISA-reactivity towards both antigens can be used to distinguish between serotype I and II FCoV infections in cats. Therefore, a subset of FCoV-positive feline sera validated for type I or type II specificity by VN assays, were cross-tested in an indirect ELISA format using FCoV serotype I and II S1 proteins as antigens (Zhao et al., unpublished observations; see **Box 1**). The data indicate that the differentiation between FCoV type I and type II infection could be achieved for 94.7% (109/115) of the FCoV positive samples based on the calculation of the S1 ELISA-based antibody titer ratio calculated for both antigens (**Box 1)**.

Box 1. Serological differentiation of FCoV infections by antibody titer ratio calculation of anti-FCoV serotype I S1: anti-FCoV serotype II S1 ($R_{1:2}$). (A) Overall distribution of ELISA titers against FCoV serotype I or II S1. While the overall distribution of ELISA titers is similar, the mean value of serotype II S1 ELISA titers is around 2.5 logs lower than of serotype I S1. (B) Correlation of virus neutralization (VN) titers of different serum samples. FCoV serotype I-specific sera, serotype II-specific sera and field cat sera are indicated in red, green and black dots, respectively. The number of seropositive samples are indicated for each VN titer. Several serotype I-specific sera as determined by VN assay also react with serotype II S1, and vice versa. (C) Correlation of titer ratio of $R_{1:2}$ with VN ability. FCoV type-specific sera were indicated as in (B). When comparing ELISA titers of serum samples, it is noticeable that the ELISA titers against type I and type II S1 differ greatly and the titer ratio $R_{1:2}$ is often in accordance with their virus neutralizing ability. Type-specific serum samples collected from experimentally infected SPF cats all exhibit ELISA titers that are at least 10-fold higher with the corresponding S1. (D) By comparing ELISA titers and calculating the $R_{1:2}$, the subset could be divided into three groups, namely $R_{1:2}$ >1 (n= 103), $R_{1:2}$ <1 (n= 6) and $R_{1:2}$ =1 (n= 6). It was shown that samples in the $R_{1:2}$ >1 group are type I -specific determined by VN assay, while samples in the $R_{1:2}$ <1 group are type II-specific. Samples in the $R_{1:2}$ =1 group mainly show low ELISA titers close to the cut-off titer.

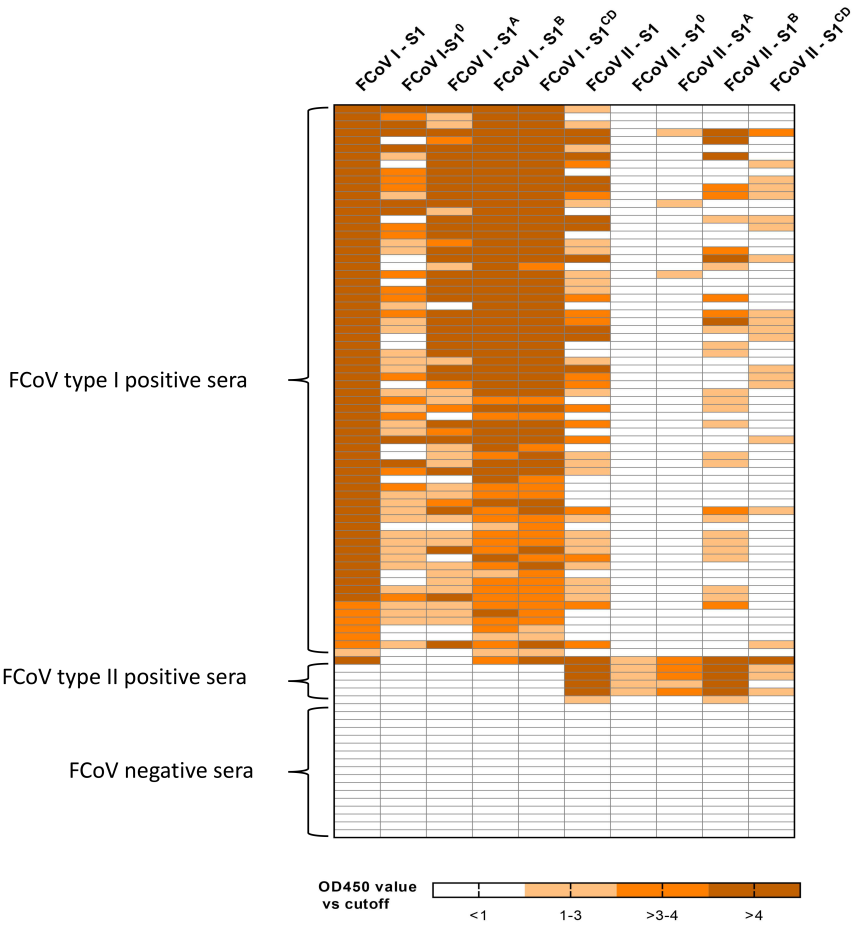


$S1^0$ and $S1^A$ are the least conserved domains and only reactive with the corresponding type-specific antisera (**Chapter 2**). To evaluate the discriminating power when using these domains as antigens, $R_{1:2}$ defined serum samples were analyzed also in an indirect ELISA format with different S1 domains, namely $S1^0$ and $S1^A$ - $S1^D$ (**Box 2**). Using these domains for ELISA screening, we observed a clear pattern of reactivity, and distinctive differences in reactivity between type I and II were noticed. Variations in antibody responses against the $S1^0$ and $S1^A$ domain were also observed within the group of FCoV type I positive sera (**Box 2**), in accordance with the lower sequence similarity found for $S1^0$ and $S1^A$ among the different S1 domains between FCoV type I S1 sequences (**Box 3**). Previous reports have also shown that variations in FCoV type I S protein, including large deletions, occur in $S1^0$ corresponding region [55,56]. Taken together, a combination of these two ELISA methods described above would allow accurate discrimination of FCoV serotype I and II infections.

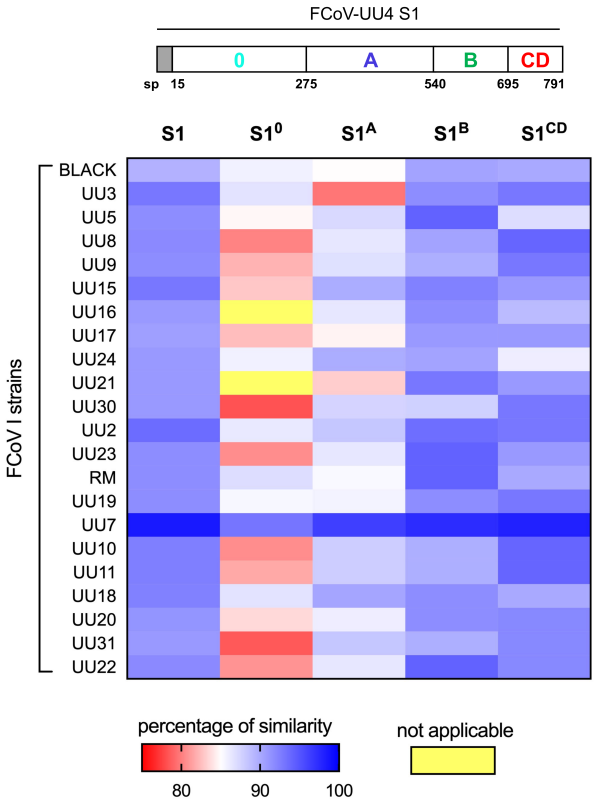
A direct approach to differentiate between CoV infections is to physically separate the antibodies against different antigens. In **Chapter 4**, we demonstrated that antibodies against canine respiratory coronavirus (CRCoV) and SARS-CoV-2 can be separated via an antigen adsorption assay. Incubation of serum samples with oversaturated quantity of one antigen allows complete removal of corresponding antibodies including the possible cross-reactive antibodies, leaving only the specific antibodies against the other antigen. Such approach was already used for serological differentiation of infection with different IAV subtypes [57]. Future studies should evaluate this approach to study cat serum samples if applicable, which will allow better differentiation between CoV infections in cats.

Cross-reactivity of different serum samples was also observed with IAV antigens. Reference antisera from immunized animals with one HA were shown to also react with HA or HA1 protein of a different subtype (**Chapter 5**). Thus, we applied three assays differing in IAV subtype-specific discriminatory power and sensitivity. The complete HA ectodomain contains HA1 and the more conserved HA2 subunit, and ELISA with HA allows detection of antibodies against epitopes present in both subunits. HA ELISA maximizes detection of IAV antibodies, including potential cross-reactive antibodies elicited by more distantly related HAs that may fail to react with HA1 but can still target the conserved HA2 subunit. In comparison, the HA1 ELISA is more type specific as HA1 is the most divergent component of HA. HA1 is also commonly used as antigen in a protein microarray format for assessing vaccination efficiency [39,40]. Lastly, the ELISA assays were complemented with our newly designed, nanoparticle-based HI assay. Through interfering HA-sialic acid interaction, this assay specifically detects antibodies targeting epitopes surrounding the HA receptor-binding site (RBS).

Box 2. ELISA reactivity of feline serum samples against different domains of FCoV serotype I or II spike protein. Relative ELISA (OD450 values divided by cut-off value) results are displayed as a heat map. Each row of the heat map represents an individual sample, and rows were arranged in a descending order based on ELISA-reactivity against FCoV serotype I S1. It is noticed that reactivities of these domains differ, with the $S1^{B}$ and $S1^{CD}$ domains showing the strongest reaction. FCoV type I positive sera react with homologous S1 domains and 50.7% (36/71) and 25.4% (18/71) of them react with $S1^{B}$ and $S1^{CD}$ of FCoV type II S1, respectively. Most of the FCoV type II positive serum samples react with all the domains of type II S1, and none of them show reactivity with type I S1 domains. Noticeably, only very limited cross-reactivity with $S1^{0}$ and $S1^{A}$ domains was observed. Three type I positive sera react with type II $S1^{A}$, while no type I positive sera showed reactivity with type II $S1^{0}$. Thus, the antibody responses detected by these two domains occur to be type specific.



Box 3. Sequence homology for the different $S1^0$, $S1^A$, $S1^B$, $S1^{CD}$ domains between FCoV type I S1 sequences are displayed as a heat map. S1 protein sequences of a group of FCoV type I strains (n=23) were aligned by the ClustalW method, and sequence similarities of different S1 domains were calculated against that of FCoV UU4 S. Schematic representation of the S1 subunit of FCoV UU4 S were presented in the top panel [54]. GenBank accession numbers for viruses have been reported previously [55]. Of note, FCoV strains UU16 and UU21 lack the $S1^0$ domain in their spike proteins, resulting from a deletion [56].



Of note, most of the serological tests that aim for increased specificity will come at a cost of sensitivity. Utilizing smaller size antigens results in recognition of less epitopes, which will inevitably narrow the range of detection. Therefore, usage of comprehensive serological assays involving different antigens is essential to allow broad specific analysis of CoV or IAV antibodies and to avoid large underestimation of viral infections in population-based seroprevalence studies.

Novel presentation of antigens for improved serology

The dissection of humoral immunity at molecular level is technically demanding. Before the advance of molecular biology, undefined mixture of antigens, such as inactivated viruses, were mainly used for serological assays. Nowadays, prokaryotic or eukaryotic expression of recombinant proteins with various modifications are attainable, which allows large quantity production of antigens, and further advanced engineering for antigen presentation. In virus particles, viral envelope proteins are ubiquitously distributed on the viral membrane. To this end, studies have focused on the making of

"virus mimics", i.e. multivalent presentation of viral proteins on sphere-shaped particles representing the size of viruses. Utilization of such "virus mimics" is proven to drastically enhance the affinity of viral protein-receptor interaction, which enables identification of novel functions of different viral proteins [58–60].

In **Chapter 5**, we developed a protein-based, virus culture free HI assay by multivalently present spy-tagged HA protein on self-assembling mi3 particles. Nanoparticles displaying HA (HA-NPs) strongly agglutinate human erythrocytes with hemagglutination titers akin to that of IAV virion. Compared to assays conducted with live or inactive virus particles, this approach circumvents the virus propagation step, for which biosafety level 3 containment is required with certain IAV subtypes and enables HI-based serology for viruses that have not or cannot be isolated in cell culture. Use of recombinant protein-based assays also allows unconstrained manipulation of antigens, e.g. expression of viral proteins from tissue culture incompatible viruses, or introduction of *de novo* mutations that reduce virus fitness, and that are therefore cannot be rescued in the context of infectious viruses by reverse genetics. A similar approach was also conducted for MERS-CoV serology, where the sialic acid binding MERS-CoV S $S1^A$ domain was multivalently conjugated on lumazine synthase nanoparticles to perform an HI assay [61]. As hemagglutination activity was observed with many other viruses, including important human pathogens like HCoV-OC43 and influenza C virus, comparable approaches could be developed accordingly for serological studies of those viruses [62].

Driven by public health, scientific and commercial interests, new diagnostic methods for laboratory diagnosis of viral infections are continuously being developed. The urgent need for serology or antibody tests during the SARS-CoV-2 pandemic has accelerated this process. Recently, several reports have shown that cell surface associated native SARS-CoV-2 S glycoprotein is highly immunogenic, which allows the use of S in serological approaches such as flow cytometry analysis with serum samples [63,64]. In the meantime, the S1 protein was also used as target for rapid virus antigen tests such as lateral flow screening assays [65,66]. Development and validation of such approaches supports the continuous refinement of diagnostic methods, which aim for consistent and specific antibody and antigen tests for the investigation of COVID-19, and for viral diagnostics in general.

Zoonosis and reverse zoonosis of CoV and IAV: insights from serological observations

In view of the high genetic diversity as a result of the relatively high error rate of RNA polymerases combined with high frequency of RNA genome recombination, RNA viruses are prone to cause spill-over events, sporadically resulting in successful cross-species transmission with adaptation to new hosts [67]. Such features pose a threat on global human health as well as animal health. CoV and IAV, the viruses studied in this thesis, are known for their ability to recombine or reassort, which contribute to their worldwide distribution and colonization of many different host species [8,9,23,24]. All CoVs able

to infect humans were shown to have a putative animal origin, while wild aquatic birds are the natural host reservoir for all IAVs of animals and humans [68,69]. Recent studies have conducted extensive sequencing or viromics researches in order to identify novel (reassortant) viruses in animals that may cause spill-over events [70,71]. As a complementary approach, serological studies allow tracing of past infections, which could serve as an indicator of novel viral infections. Thus, applying a set of serological methods, we were able to trace previous CoV and IAV infections of companion animals, and estimate the (reverse) zoonosis events that might have occurred.

In **Chapter 2**, we provided serological evidence that infections in cats with endemic FCoV are highly prevalent, while we also identified antibodies against HCoV-229E and PDCoV S1 in feline serum samples, including samples that reacted solely with either antigen. Both HCoV-229E and PDCoV utilize aminopeptidase N (APN) as receptor and were shown able to use feline APN for cellular entry [72–74]. HCoV-229E is an endemic human CoV that can cause the common cold [68]. Prior inoculation with HCoV-229E in cats primes FCoV antibody responses upon FCoV infection, which support susceptibility of cats for HCoV-229E infection [75]. Therefore, the unique seropositivity identified against HCoV-229E S1 in some samples is probably due to natural exposure to HCoV-229E through reverse zoonosis via daily human contact. PDCoV is a CoV discovered less than ten years ago, with a reputed avian origin [76]. As cats are natural avian predators, it is possible that encounter with PDCoV or PDCoV-related CoVs induced the seroreactivity that was detected. Interestingly, transmission of PDCoV is similar to swine flu, where wild aquatic birds are the natural reservoir, and pigs are associated as an intermediate host reservoir for (possible) future virus dispersal [23,77]. Another striking fact is the promiscuity of PDCoV S. A recent study by Li *et al.* showed that PDCoV S is able to bind a highly conserved site present on APN of various hosts including human, allowing cellular entry of PDCoV [74]. The global distribution in pigs and the apparent receptor promiscuity marks PDCoV as a virus with zoonotic and pandemic potential and warrants the need for serological studies in humans to monitor spill-over infections.

A more recent and convincing indication of CoV reverse zoonosis is shown with SARS-CoV-2. Since its emergence in December 2019, sporadic detection of natural SARS-CoV-2 infection in certain animals, as well as successful experimental inoculation in different animal models were conducted [18,78]. Such observations have raised the concern regarding reverse zoonosis and subsequent secondary zoonotic events, namely transmission of the virus from infected animals back to humans. In a recent report, it has been confirmed that workers from mink farms had acquired SARS-CoV-2 from minks, the victims of reverse zoonosis [79]. Focusing on SARS-CoV-2 infection in cats and dogs, in **Chapter 4** we established a set of serological assays that allow detection of SARS-CoV-2-specific antibodies in Dutch cats and dogs. We showed that the general SARS-CoV-2 seroprevalence is low in cats and dogs with unknown virus exposure, but the seropositive rate in SARS-CoV-2 exposed animals

is high (24.4%). Our finding is in agreement with other serological studies of a similar time period, where high seroprevalence was also determined in companion animals in severely SARS-CoV-2 affected geographic areas [80,81]. There is currently no evidence that pets may play a role in the transmission of the virus. The fact that cats and dogs are susceptible to SARS-CoV-2 and their potent antibody response post COVID-19 exposure draws the concern of them being possible reservoirs for secondary zoonosis, and supports the need for far-reaching and continuous surveillance of SARS-CoV-2 in pets.

Although the world's focus is currently on SARS-CoV-2, IAV remains to be a notorious pathogen posing zoonotic threats of (recurrent) pandemic potential [77]. Cats and dogs are recognized as IAV hosts, but comprehensive serological methods suitable for cats and dogs, as well as knowledge on circulation of IAV in cat and dog population in Europe, are still lacking. In **Chapter 5**, we analyzed antibody responses in serum samples from various Dutch cat and dog cohorts against HA proteins of different IAV subtypes through a set of newly established serological assays. It was noticed that the majority of responses was constituted by antibodies against human-derived pandemic H1N1 (H1N1pdm09). Samples collected before 2009 do not share the same H1N1pdm09 seropositive ratio, supporting the occurrence of reverse zoonosis events during and after the 2009 H1N1 pandemic. It is likely that reverse zoonosis with H3N2 seasonal IAV also occur, as shown by positive antibody responses against H3N2 HA. In the meantime, we provide the first serological evidence of infection of avian subtype IAV in cats (H3, H5, H7, and H9) and dogs (H5 and H7) in Europe. Likely, this seropositivity is due to spillover of avian subtype IAVs from birds to cats and dogs, partly because of their predatory behavior. It is of low but not to be neglected risk that cats and dogs might transmit avian subtype IAVs to humans, as corroborated by the zoonotic infection by feline H7N2 virus in 2017 [82,83]. Taken together, these observations emphasize the need for recurrent epidemiological surveillance for influenza infections in cats and dogs, which functions as an early warning system for human and animal threats.

Concluding remarks

CoVs and IAVs are well known for their ability to jump the host species border. To get insight into the frequency of interspecies transmission of CoVs and IAVs within and between animal and human populations and to understand the potential risk of subsequent development of a pandemic, it is useful to screen for CoVs and IAVs infections in animal species; especially those that are in close contact with humans. Serological assays detect virus-specific antibody responses against infection, and subsequently play an important role in the understanding of CoVs and IAVs epidemiology. In this thesis, we presented comprehensive serological methods for endemic animal CoVs, SARS-CoV-2 and IAV. By using these methods, we were able to trace past infections in companion animals and evaluate the risk of zoonosis and reverse zoonosis. Although the assays developed here were only applied to animal samples, it would be of relevance for serology with human samples, especially with the methods developed for SARS-CoV-2 and IAV. Our observations underline the role of companion animals in CoV and IAV ecology, and the potential risk of them to give rise to novel recombinant viruses.

The frequency with which CoV and IAV epidemics and pandemics occurred in the past suggests that future outbreaks of novel CoV and IAV in humans are inevitable. To foresee such threats and estimate the risk on animal or human health, recurrent surveillance studies are needed. In multiple stages of an epidemic, serological studies can assist in understanding of spread of infections. In this prospective, our work adds to the increasing consideration of using comprehensive serological methods to monitor virus prevalence and emergence.

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6

Addendum

Nederlandse Samenvatting

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Curriculum Vitae

List of Publications

Nederlandse Samenvatting

Coronavirussen (CoV's) en influenza A-virussen (IAV's) staan bekend om hun vermogen barrières van gastheersoorten te overschrijden en een breed scala aan vogelsoorten en zoogdiergastheersoorten, waaronder mensen, te infecteren. Hun mogelijke opkomst na zoönotische introductie vormt een aanzienlijke bedreiging voor de volksgezondheid. Iets meer dan een eeuw geleden trof de grieppandemie van 1918, veroorzaakt door een H1N1 IAV van aviaire oorsprong, een derde van de wereldbevolking en leidde tot meer dan 50 miljoen doden. Enkele decennia later, in de jaren 1957, 1968 en 2009, deden zich drie andere grote IAV-pandemieën voor, die elk ernstige gevolgen hadden voor de wereldeconomie en de volksgezondheid. In de tussentijd kwamen, naast de gevestigde menselijke coronavirussen, CoV-uitbraken bij mensen alleen al in de 21e eeuw drie keer voor: de ernstige acute respiratoire syndroom (SARS) CoV-uitbraak in 2002/2003, het Midden-Oosten respiratoir syndroom CoV in 2012 en de momenteel heersende SARS-CoV-2-pandemie. Gezelschapsdieren zijn ook vatbaar voor CoV's en IAV's. Naast de gevolgen voor de diergezondheid, brengt het nauwe contact tussen gezelschapsdieren en mensen ook potentiële risico's van zoönotische virusinfecties met zich mee, en is bij deze dieren ook omgekeerde zoönose van menselijke CoV's en IAV's waargenomen. Dergelijke feiten benadrukken ook de rol van gezelschapsdieren als potentiële virusmengvaten, met de mogelijkheid van de opkomst van recombinante virussen met verhoogde bedreigingen voor zowel dier als mens. Daarom is het van belang om CoV- en IAV-infecties bij gezelschapsdieren te bestuderen, en zijn regelmatige surveillancestudies noodzakelijk.

Klassiek gedefinieerd als de studies van eiwitten die worden aangetroffen in lichaamsvloeistoffen, vooral bloedserum of plasma, werd serologie vaak gebruikt om de prevalentie van pathogeen-specifieke antilichamen in gedefinieerde populaties te bestuderen. Vergeleken met moleculaire diagnostische methoden in het acute stadium van infectie, hebben serologische methoden het voordeel dat de infectie na een langere periode kan worden opgespoord, aangezien door virussen geïnduceerde antilichamen na infectie een lange persistentie in het bloed hebben. Daarom zijn serologische testen cruciale instrumenten die kunnen worden toegepast ter ondersteuning van de klinische diagnose van een virale infectie, het monitoren van de naleving van vaccinatie, beoordelingen van de populatie immuniteit en seroprevalentiestudies in verschillende populaties. In dit proefschrift hebben we ons gericht op de ontwikkeling van robuuste en specifieke serologische testen die kunnen dienen als toolkit voor een snelle diagnose van CoV- en IAV-infecties. Dit zal meer inzicht en kennis opleveren over het voorkomen van verschillende CoVs en IAVs-infecties bij gezelschapsdieren en hun associatie met klinische verschijnselen.

Bij katten komen infecties met feline coronavirussen (FCoV's) vaak voor. Van verschillende niet-katachtige coronavirussen is vastgesteld dat ze zowel kattencellen als katten kunnen infecteren na experimentele infectie, ondersteund door hun vermogen om de katachtige receptor ortholoog te gebruiken voor infectie van de cel. Het is echter niet bekend of natuurlijke infecties met CoV's van andere soorten bij de kat kunnen voorkomen. In Hoofdstuk 2 presenteerden we een grondig serologisch onderzoek bij katten gebruik makende van de S1-receptorbindende subeenheid van het CoV-spike-eiwit, dat immunogeen is en een lage aminozuursequentie-identiteit heeft onder coronavirus-soorten. Recombinante CoV-spike S1-eiwitten van verschillende dierlijke en menselijke CoV's werden gebruikt als antigenen voor het screenen van kattensera op de aanwezigheid van antilichamen tegen de respectievelijke eiwitten. Positieve monsters werden verder getest door middel van virusneutralisatietesten. We hebben antigene kruisreactiviteit waargenomen tussen S1's van type 1 en type 2 FCoV's, en tussen FCoV type 1 en porcine epidemische diarree-virus. Het in kaart brengen van domeinen van antilichaamepitopen duidde op de aanwezigheid van geconserveerde epitopen, in het bijzonder in de CD-domeinen van S1. Onze waarneming dat sommige katachtige sera antilichaamreactiviteit vertoonden uitsluitend tegen niet-katachtige CoV S1-eiwitten rechtvaardigt verder onderzoek naar de epidemiologie en de overdracht tussen soorten van coronavirussen bij katten en andere dieren die in nauw contact staan met mensen.

Epidemiologische onderzoeken naar infectie met het equine coronavirus (ECoV) zijn nog beperkt, en de seroprevalentie van ECoV-infectie in Europa is onbekend. In Hoofdstuk 3 wordt de ontwikkeling en validatie van een op S1-proteïne gebaseerde ELISA-methode voor de detectie van specifieke antilichamen tegen ECoV beschreven. Met deze methode zijn we in staat om een geconsolideerde diagnostische test te leveren om ECoV-uitbraken te bevestigen, als aanvulling op de qRT-PCR-analyse van uitwerpselen van paarden. Onze in-house ELISA geeft betrouwbare diagnostische prestaties in vergelijking met de VN-test en is een nuttige test om seroconversie te ondersteunen bij paarden die betrokken zijn bij ECoV-uitbraken en om de ECoV-seroprevalentie in populaties van paarden te bepalen.

In Hoofdstuk 4 hebben we een onderzoek uitgevoerd om de verspreiding van SARS-CoV-2 infecties bij gezelschapsdieren te volgen en te bestuderen. Een reeks serologische testen, waaronder ELISA en virusneutralisatie, werd ontwikkeld en gevalideerd en vervolgens gebruikt om het eerste seroprevalentieonderzoek in Nederland uit te voeren. De algemene prevalentie van antilichamen tegen SARS-CoV-2 binnen dit onderzoek in de dierenpopulatie met onbekende SARS-CoV-2 is laag, maar ondersteunt het belang van continue serosurveillance van SARS-CoV-2 bij deze gezelschapsdieren en een breder scala van andere diersoorten. Dit is vooral belangrijk wanneer de incidentie van COVID-19 bij mensen in verschillende delen van de wereld afneemt.

Behalve voor studies met CoV's, hebben we in Hoofdstuk 5 een reeks van serologische testen ontwikkeld die een brede tot specifieke analyse van IAV-specifieke antilichaamresponsen bij katten en honden mogelijk maakt. In deze reeks (pijplijn) werden serummonsters eerst getest met HA- en HA1-specifieke ELISA's en vervolgens geanalyseerd met op nanodeeltjes gebaseerde, virusvrije HI-assays. Met behulp van deze drie assays hebben we gevonden dat katten- en hondensera uit verschillende cohorten positief zijn voor antilichamen tegen een of meer IAV-subtypen en / of -stammen. Serummonsters van katten en honden die na de pandemische H1N1-uitbraak van 2009 werden verzameld, vertonen een veel hoger aantal seropositiieven tegen H1 vergeleken met monsters van vóór 2009. Kattensera vertoonden bovendien een hogere reactiviteit voor aviaire IAV's dan hondensera. Onze bevindingen tonen de toegevoegde waarde aan van het gebruik van complementaire serologische assays, die zijn gebaseerd op reactiviteit met verschillende aantallen HA-epitopen, om IAV-antilichaamresponsen te bestuderen en voor verbeterde serosurveillance van IAV-infecties. Deze waarnemingen benadrukken de rol van katten en honden in de ecologie van IAV en duiden op het potentieel van deze gezelschapsdieren als bron van nieuwe (opnieuw gesorteerde) virussen met verhoogd zoönotisch potentieel.

In meerdere stadia van een epidemie kunnen serologische onderzoeken helpen om de verspreiding van infecties te begrijpen. In deze zin draagt ons werk bij aan de toenemende overweging om uitgebreide serologische methoden te gebruiken om de prevalentie en opkomst van virussen te controleren.

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Shan Zhao (赵珊)

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Curriculum Vitae

Shan Zhao was born on April 19th, 1992 at Ya'an, Sichuan province, P.R. China. She enrolled in Sichuan Ya'an Middle school in 2007. After receiving her middle school diploma in 2010, she studied her bachelor's program in veterinary medicine at the college of veterinary medicine, Sichuan Agricultural University in Ya'an, Sichuan province. After receiving her bachelor's degree in 2014, she continued her master study in Sichuan Agricultural University, focusing on preventive veterinary medicine. Her MSc thesis was about the sensitive detection of Porcine circovirus-2 by droplet digital PCR. She obtained her master's degree in June 2016. In April 2017, as a PhD candidate she began her further research in Virology Section, Infectious Diseases and Immunology Division, Department of Biomolecular Health Sciences, Faculty of Veterinary Medicine, Utrecht University, the Netherlands. Her PhD project is partially funded by the China Scholarship Council. Under the supervision of Dr. Herman Egberink, Dr. Berend-Jan Bosch and Prof. Dr. Frank J.M. van Kuppeveld, her research project focused on seroepidemiological studies of coronavirus and influenza A virus infections in companion animals, and the results of this project are presented in this thesis.

List of publications

1. **Zhao S,** Schuurman N, Li W, Wang C, Smit LAM, Broens EM, Wagenaar JA, van Kuppeveld FJ, Bosch BJ, Egberink H. Development and application of a toolbox for serological screening of SARS-CoV-2 infection in cats and dogs during the first COVID-19 wave in the Netherlands. ***Emerging Infectious Diseases. In press***

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3. **Zhao S**, Smits C, Schuurman N, Barnum S, Pusterla N, Van Kuppeveld F, Bosch BJ, Van Maanen K, Egberink H. Development and validation of a S1 protein-based ELISA for the specific detection of antibodies against equine coronavirus. ***Viruses***. 2019 Dec;11(12):1109.

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