

1 **Title: Multi-species ELISA for the detection of antibodies against SARS-CoV-2 in animals**

2 **Running title: Multi-species ELISA for SARS-CoV-2 antibodies**

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16 **Summary**

17 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused a pandemic with millions of
18 infected humans and hundreds of thousands of fatalities. As the novel disease - referred to as COVID-19 -
19 unfolded, occasional anthrozoootic infections of animals by owners or caretakers were reported in dogs,
20 felid species and farmed mink. Further species were shown to be susceptible under experimental conditions.
21 The extent of natural infections of animals, however, is still largely unknown. Serological methods will be
22 useful tools for tracing SARS-CoV-2 infections in animals once test systems are validated for use in different
23 species. Here, we developed an indirect multi-species ELISA based on the receptor-binding domain (RBD) of
24 SARS-CoV-2. The newly established ELISA was validated using 59 sera of infected or vaccinated animals
25 including ferrets, raccoon dogs, hamsters, rabbits, chickens, cattle and a cat, and a total of 220 antibody-
26 negative sera of the same animal species. Overall, a diagnostic specificity of 100.0% and sensitivity of 98.31%
27 was achieved, and the functionality with every species included in this study could be demonstrated. Hence,
28 a versatile and reliable ELISA protocol was established that enables high-throughput antibody detection in a
29 broad range of animal species, which may be used for outbreak investigations, to assess the seroprevalence
30 in susceptible species or to screen for reservoir or intermediate hosts.

31

32 **Keywords:** SARS-CoV-2; COVID-19; diagnostics; serology; animals; felines; mustelids; raccoon dog; cattle;
33 chicken

34 Introduction

35 Since the beginning of 2020, the acute respiratory disease COVID-19 caused by a novel betacoronavirus,
36 severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Zhu et al., 2020), has been keeping the world
37 in suspense. COVID-19 emerged for the first time in December 2019 in Wuhan, China (WHO, 2020b), and has
38 developed rapidly into a global pandemic (WHO, 2020a) resulting in millions of infections and hundreds of
39 thousands of deaths. The symptoms in affected humans range from inapparent infections to fever, fatigue,
40 cough, shortness of breath or severe pneumonia and death (WHO, 2020c). SARS-CoV-2 is primarily
41 transmitted between infected humans through droplets and fomites (Ghayda et al., 2020; WHO, 2020c).
42 However, the virus is thought to originate from an animal reservoir, from where it was likely transmitted to
43 humans by either a direct spill-over event presumably followed by natural selection and adaptation in
44 humans, or via an intermediate mammalian host (Andersen, Rambaut, Lipkin, Holmes, & Garry, 2020). As
45 SARS-CoV-2 is highly similar to bat betacoronaviruses (Latinne et al., 2020; Wu et al., 2020; Zhou et al., 2020)
46 or betacoronaviruses found in pangolins (Zhang, Wu, & Zhang, 2020), it is suspected that one of those animal
47 species may represent the original host of the SARS-CoV-2 ancestral virus. Considering the presumed
48 zoonotic origin of SARS-CoV-2 and the fact that highly similar orthologues of the human angiotensin-
49 converting enzyme (ACE2) receptor are present in certain animal species (Li, 2013; Sun, Gu, Ma, & Duan,
50 2020), it is of interest to identify susceptible animal species and to assess SARS-CoV-2 prevalence in these
51 species. As the COVID-19 pandemic unfolded, occasional infections of dogs, domestic and non-domestic felid
52 species (cat, tiger, lion) and farmed minks were reported (Enserink, 2020; McAloose et al., 2020; Oreshkova
53 et al., 2020; Sailleau et al., 2020; Sit et al., 2020; Wang et al., 2020). The majority of SARS-CoV-2-infections in
54 animals were directly linked to SARS-CoV-2-infected owners or animal caretakers. Under experimental
55 conditions, the susceptibility of additional species including ferrets, hamsters, and fruit bats has been
56 demonstrated (Osterrieder et al., 2020; Richard et al., 2020; Schlottau et al., 2020; Shi et al., 2020). However,
57 the level of natural infections is largely unknown. Currently, infection in animals is mostly confirmed by direct
58 virus detection using (real-time) RT-PCRs. While this is highly sensitive and specific, experimental and field
59 data demonstrate that animals test positive only during a very short time interval. Therefore, to elucidate the
60 host range of SARS-CoV-2 and the prevalence in susceptible species serological assays, i.e. multi-species

61 antibody detection systems, could be beneficial. Serum neutralization tests, which are species-independent,
62 rely on *in-vitro* interaction between the infectious virus and specific antibodies and, therefore, require high-
63 containment laboratories to handle the virus. In contrast, enzyme-linked immunosorbent assays (ELISAs) can
64 be applied under less stringent biosafety conditions, once such test systems are validated for use in animals.
65 In addition, ELISAs enable high-throughput testing of clinical specimens.
66 For human sera, a wide range of ELISA systems have been developed within a very short time based on
67 different antigens that include the viral nucleocapsid (N) protein, the spike protein (S) or the S receptor-
68 binding domain (RBD) (Beavis et al., 2020; Klumpp-Thomas et al., 2020; Tré-Hardy et al., 2020). When
69 comparing different antigens as assay targets, considerable cross-reactivity between different coronaviruses
70 occurred in whole-virus or N-based serological assays, while S or RBD based protocols demonstrated a much
71 higher specificity (Chia et al., 2020; Klumpp-Thomas et al., 2020; Meyer, Drosten, & Müller, 2014). Based on
72 these findings, we selected different domains of the S protein for the development of a multi-species SARS-
73 CoV-2 antibody ELISA to avoid cross-reactivity and associated non-specificity. This is especially important as
74 coronaviruses including several betacoronaviruses are widespread in animals (Amer, 2018; Drechsler,
75 Alcaraz, Bossong, Collisson, & Diniz, 2011; Erles & Brownlie, 2008; Hodnik, Ježek, & Starič, 2020; Murray,
76 Kiupel, & Maes, 2010; Tizard, 2020), and could potentially cross-react in SARS-CoV-2 test systems.

77

78 **Materials and methods**

79 Protein expression and ELISA procedure

80 For expression of the SARS-CoV-2 S1 and the RBD-SD1 domain, amino acids (aa) 17 to 685 or 319 to 519,
81 respectively, were amplified from a codon-optimized synthetic gene (GeneArt synthesis, ThermoFisher
82 Scientific, Germany). The constructs were cloned into the pEXPR103 expression vector (iba lifesciences,
83 Germany) in-frame with an N-terminal modified mouse IgK light chain signal peptide and a C-terminal double
84 Strep-tag. Expi293 cells were grown in suspension in Expi293 expression medium (ThermoFisher Scientific) at
85 37 °C, 8 % CO₂, and 125 rpm. The cells were transfected using the ExpiFectamine293 transfection kit
86 (ThermoFisher Scientific) according to the manufacturer's instructions. Five to six days after transfection, the
87 supernatants were harvested by centrifugation at 6000xg for 20 min at 4 °C. Biotin was blocked by adding

88 BioLock (iba lifesciences) as recommended, and the supernatant was purified using Strep-Tactin XT
89 Superflow high capacity resin (iba lifesciences) according to the manufacturer's instructions. Finally, the
90 proteins were eluted with 50 mM biotin (in 100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA; pH 8.0). The
91 expression of the proteins was verified by SDS-PAGE and western blotting using a HRP-conjugated anti-Strep-
92 tag antibody (dilution 1/20,000, iba lifesciences). Reactive bands were visualized using the Super Signal West
93 Pico Chemiluminescent substrate (ThermoFisher Scientific) and an Intas ChemoCam system (Intas Science
94 Imaging Instruments GmbH, Germany).

95 For the ELISA, medium-binding plates (Greiner Bio-One GmbH, Germany) were coated with 100 ng/well of
96 either the RBD or S1 antigen overnight at 4 °C in 0.1 M carbonate buffer (1.59 g Na₂CO₃ and 2.93 g NaHCO₃,
97 ad. 1 l aqua dest., pH 9.6), or were treated with the coating buffer only. Thereafter, the plates were blocked
98 for 1 h at 37 °C using 5 % skim milk in phosphate-buffered saline (PBS). To evaluate the optimal
99 concentration, sera were pre-diluted 1/50, 1/100 or 1/200 in Tris-buffered saline with Tween 20 (TBST), and
100 incubated on the coated and uncoated wells for 1 h at room temperature. A multi-species conjugate
101 (SBVMILK; IDvet, France) was diluted 1/40 or 1/80 and then added for 1 h at room temperature. For chicken
102 samples, an anti-chicken conjugate (Rabbit anti-Chicken IgY (H+L) Secondary Antibody, HRP; ThermoFisher
103 Scientific) diluted 1/10,000 was applied. Following the addition of tetramethylbenzidine (TMB) substrate
104 (IDEXX, Switzerland), the ELISA readings were taken at a wavelength of 450 nm on a Tecan Spectra Mini
105 instrument (Tecan Group Ltd, Switzerland). Between each step, the plates were washed three times with
106 TBST. The adsorbance was calculated by subtracting the optical density (OD) measured on the uncoated
107 wells from the values obtained from the protein-coated wells for the respective sample.

108

109 Serum samples

110 A total of 220 animal sera (51 cat, 33 cattle, 32 ferret, 27 raccoon dog, 35 chicken, 30 rabbit, 12 hamster)
111 collected before the SARS-CoV-2 pandemic or representing pre-infection sera of SARS-CoV-2 animal studies
112 were included as negative control samples. The SARS-CoV-2 negative status of the latter was verified by a
113 virus neutralization test (VNT) or in an indirect immunofluorescence assay (iIFA).

114 Eight ferret, 20 hamster, 16 raccoon dog and two cattle SARS-CoV-2 antibody-positive sera originated from
115 experimental infection studies and were collected between 8 and 28 days post infection (dpi) (Freuling et al.,
116 2020; Osterrieder et al., 2020; Schlottau et al., 2020; Ulrich, Wernike, Hoffmann, Mettenleiter, & Beer, 2020;
117 Balkema-Buschmann, Rissmann et al., unpublished). With the exception of the bovine samples, all sera
118 tested positive in a VNT, in an iIFA, or in both, with the iIFA being more sensitive for antibody detection early
119 after infection (Schlottau et al., 2020). The cattle sera were collected 12 and 20 dpi from the same animal.
120 The first sample tested negative by iIFA, while the second showed weak positive signals (titer 1/4). In
121 addition, three sequential sera from a naturally SARS-CoV-2 infected cat were included (ProMED-mail, 2020).
122 The titers of the feline samples as measured by VNT were 1/40 (serum collected 15 days after the first SARS-
123 CoV-2 positive throat swab sample), 1/51 (22 days) and 1/40 (29 days), respectively. Furthermore, sera of
124 three chickens (49 days post prime and 28 days post boost immunization, iIFA positive >1/640) and two
125 rabbits immunized with the RBD and three chickens (49 days post prime and 28 days post boost
126 immunization, iIFA inconclusive) and two rabbits immunized for immuno-serum generation with the S1
127 protein were added.

128

129 Sensitivity, specificity, repeatability and reproducibility

130 To determine the cut-off values and the diagnostic sensitivity and specificity of the final ELISA protocol, the
131 sera were tested, and receiver operating characteristic (ROC) analyses were performed.

132 For evaluation of intra-assay reproducibility, a negative and a very weak-positive cattle serum (collected at
133 12 dpi) as well as a positive raccoon dog serum (16 dpi) were tested in five replicates each. The inter-assay
134 repeatability was determined with the identical samples and replicate numbers on five independent ELISA
135 plates. Mean values and standard deviations of the 25 replicates were calculated. All analyses and
136 visualizations were performed using GraphPad Prism version 8.0 for Windows (GraphPad Software, USA).

137

138 **Results and Discussion**

139 Both SARS-CoV-2 protein domains, i.e. RBD and S1, were successfully expressed (figure 1), and their
140 functionality was proven by their reactivity with sera from infected or immunized animals (figures 2 and 3).

141 Thereafter, both proteins were compared regarding the optimal conditions for their use as capture antigens
142 in antibody ELISAs. In general, a higher absorption signal was obtained for positive control sera when the
143 RBD domain was coated, independent of the applied serum or conjugate concentration, while S1 as target
144 antigen led to a stronger background signal for negative control samples in every approach (figure 2). It has
145 been reported in previous studies that the RBD domain is the most specific antigen for the detection of SARS-
146 CoV-2 antibodies in humans, predominantly because this region is a major target of antibodies, and, in
147 addition, is poorly conserved between different coronaviruses (Amanat et al., 2020; Chia et al., 2020;
148 Premkumar et al., 2020). Considering the results of the RBD/S1 comparison in this study, the same holds true
149 for animals. Alternatively, the superior performance of the RBD domain might be related to the protein
150 production and purification procedure, a phenomenon that has been already described for SARS-CoV-2
151 proteins (Klumpp-Thomas et al., 2020). While the expression of the RBD is relatively straightforward, the
152 production of the large-size, heavily glycosylated S1 domain is more challenging. Both, expression and
153 purification are inefficient and consequently require pooling of several independently produced batches and
154 more hands-on-time, which might lead to a lower quality of the final protein preparation. Here, we selected
155 the RBD domain for further use because of the stronger specific signal combined with a lower unspecific
156 background signal.

157 When assessing different sample and conjugate concentrations on the RBD-coated plates, an optimized
158 signal-to-noise ratio was achieved by using serum and multi-species conjugate dilutions of 1/100 and 1/80,
159 respectively (figure 2). Hence, all following analyses were carried out using this test setup.

160 In order to evaluate both the sensitivity and specificity of the final test protocol and to establish a threshold
161 for positivity, SARS-CoV-2 antibody-negative and -positive sera of multiple species were tested. ROC curve
162 analyses indicated that the ELISA had a very high diagnostic accuracy with an area under the curve (AUC) of
163 0.989 (95% confidence interval (CI): 0.969 to 1.00). Based on the ROC curve (figure 3A), a cut-off of ≤ 0.2 for
164 negativity and ≥ 0.3 for positivity was set, with the intermediate zone between 0.2 and 0.3 being inconclusive.
165 Using these cut-off values, an overall diagnostic specificity of 100.0% (95% CI: 98.34% to 100.0%) and
166 sensitivity of 98.31% (95% CI: 90.91% to 99.96%) was achieved, where the optimal specificity is particularly
167 remarkable, as in some of the animal sera high titers of antibodies against non-SARS beta-coronaviruses

168 were present like bovine coronaviruses (Ulrich, Wernike, Hoffmann, Mettenleiter, & Beer, 2020).

169 Consequently, the previously described high suitability and accuracy of RBD-based antibody detection
170 systems for human coronaviruses (Chia et al., 2020; Meyer et al., 2014) could also be confirmed for different
171 animal species.

172 Regarding the diagnostic sensitivity, every antibody positive serum included in this study could be identified,
173 with only one exception (figure 3C). This was a single putative seropositive sample that was collected from a
174 ferret 12 days after experimental infection, which tested negative by VNT and exhibited a low titer of 1/64 in
175 the iIFA (ferret number 5 described in (Schlottau et al., 2020)). When considering only sera collected later
176 than 12 days after infection, every individual was correctly detected as being antibody-positive, independent
177 of the animal species. Thus, the newly established ELISA protocol enables the antibody detection in a broad
178 range of animal species that are susceptible to SARS-CoV-2, such as ferrets, cats, raccoon dogs, hamsters or
179 cattle. Furthermore, the applicability could be shown for further species like rabbits or chicken (figure 3C),
180 which are frequently used for reagent production for research, diagnostics and therapeutic purposes
181 (Farzaneh, Hassani, Mozdziak, & Baharvand, 2017; Popkov et al., 2003; Rossi et al., 2005). Hence, the
182 versatility was demonstrated for a test system that does not require high-containment laboratories as e.g.
183 species-independent neutralization assays. However, when further species not included in the current
184 validation are to be tested, the test has to be validated including the suitability of the conjugate for the
185 particular species in question. Alternatively, a species-specific conjugate may be used, as shown here for
186 chicken (figure 3).

187 In the final step of the validation, both the repeatability and reproducibility were assessed using five
188 replicates each of three sera in five independent ELISA assays. The negative cattle serum and the positive
189 raccoon dog sample tested correctly in each case with very low variations (figure 3B). Mean OD values and
190 standard deviations of 0.03 ± 0.02 and 0.72 ± 0.07 were calculated for the antibody-negative and -positive
191 sample, respectively. Every replicate of the very weak positive cattle sample tested positive or resulted in the
192 doubtful measuring range of the ELISA (mean 0.28, standard deviation 0.03, min 0.21, max 0.33). Thus, the
193 animal would have been detected as SARS-CoV-2-antibody positive in every approach (figure 3B), further
194 demonstrating the reliability of the test system.

195 In conclusion, we established a versatile ELISA protocol for the highly sensitive and specific detection of
196 SARS-CoV-2 antibodies in animal sera, which could be used for outbreak investigations, monitoring studies or
197 to identify yet unknown reservoir or intermediate hosts.

198

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202 **Conflict of Interest:** None.

203 **Data availability statement:** The data that support the findings of this study are available from the
204 corresponding author upon reasonable request.

205 **Ethical Statement:** The antibody negative sera represented routine diagnostic submissions taken by the
206 responsible veterinarians in the context of health monitoring (no permissions were necessary to collect the
207 specimens), or originated from unrelated animal trials, which were reviewed by the responsible state ethics
208 commission and were approved by the competent authority (State Office of Agriculture, Food Safety, and
209 Fishery in Mecklenburg-Western Pomerania). The SARS-CoV-2 antibody positive specimens were collected in the
210 context of infection trials, monitoring of the natural course of infection, or immunizations conducted for
211 diagnostic reagent production (permission numbers MV/TSD/7221.3-2-010/18-12 and 7221.3-2-042/17 (State
212 Office of Agriculture, Food Safety, and Fisheries in Mecklenburg-Western Pomerania), 33.8-42502-05-20A522
213 (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit) and 0086/20 (Landesamt für
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329 **Figure legends**

330 **Figure 1:** Western blot analyses of purified RBD-SD1 and S1 proteins used for plate coating and/or
331 immunization.

332 **Figure 2:** Optimization of the SARS-CoV-2 ELISA. Three raccoon dog sera that tested positive by an indirect
333 immunofluorescence assay (animal numbers #7, #10 and #11) and three negative sera (#8, #9 and #12) were
334 diluted 1/50, 1/100 or 1/200, and tested in combination with a multi-species conjugate (dilution 1/40 or
335 1/80) against the SARS-CoV-2 RBD-SD1 domain (black bars) or S1 protein (grey bars) expressed in Expi293
336 cells. The serum-conjugate combination that was selected for the final ELISA protocol is framed in red.

337 **Figure 3:** Assay validation and diagnostic performance. A) Receiver operating characteristic (ROC) analyses of
338 the SARS-CoV-2 ELISA using the RBD domain as antigen and 220 negative animal sera (ferret, raccoon dog,
339 cat, cattle, chicken, rabbit and hamster) and 59 sera of infected or vaccinated animals. B) Reproducibility and
340 repeatability of the ELISA. A negative and a very weak positive cattle serum, as well as a positive sample
341 collected from a raccoon dog were tested in five replicates each in five independent approaches. The box
342 plots represent the results of all 25 respective replicates. Each outlier is marked by a circle. C) Performance of
343 the SARS-CoV-2 ELISA for animal sera from clinical trials. Samples taken on day 8 or 12 after infection are
344 shown by open squares. Sera that were collected from day 15 onwards after an experimental infection
345 (ferret, raccoon dog, cattle, hamster) or immunization (chicken, rabbit), or after the first RT-PCR positive
346 throat swab sample (cat) are indicated by filled squares. Negative control samples are shown by circles. The
347 total number of sera per group is given in brackets.

Figure 1

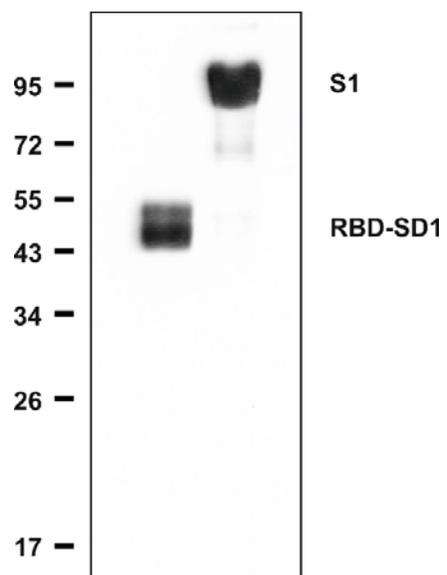


Figure 2

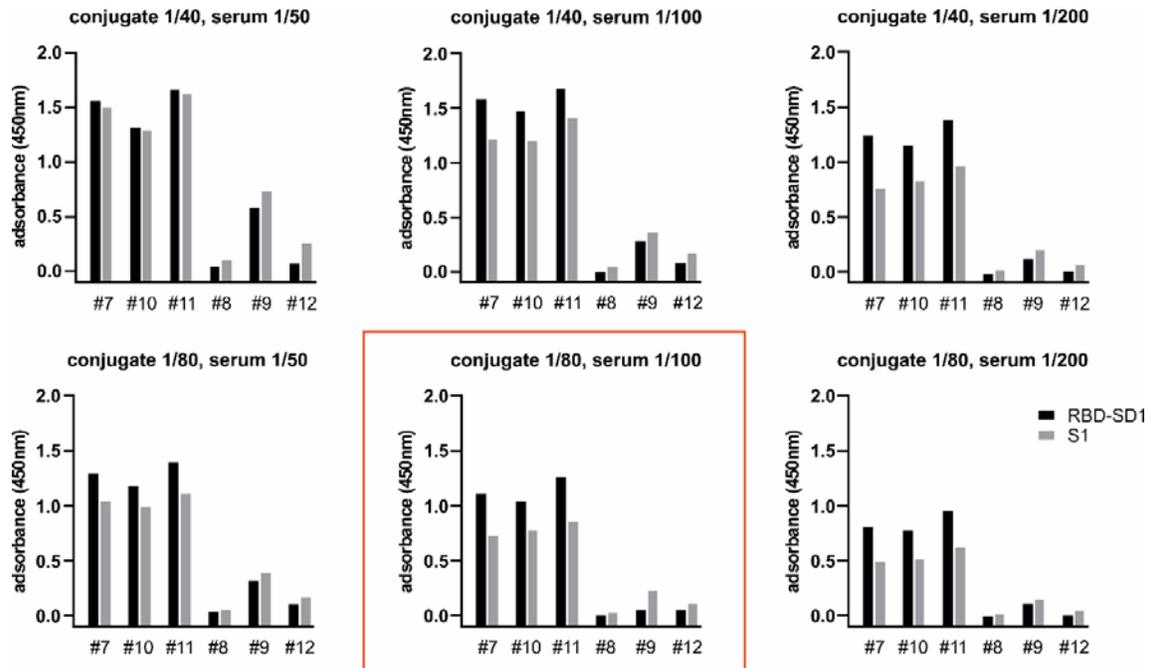


Figure 3

