Title: Predicting the presence and titer of rabies virus neutralizing antibodies from low-volume serum samples in low-containment facilities

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Summary

Serology is a core component of the surveillance and management of viral zoonoses. Virus neutralization tests are a gold standard serological diagnostic, but requirements for large volumes of serum and high biosafety containment can limit widespread use. Here, focusing on *Rabies lyssavirus*, a globally important zoonosis, we developed a pseudotype micro-neutralization rapid fluorescent focus inhibition test (pmRFFIT) that overcomes these limitations. Specifically, we adapted an existing micro-neutralization test to use a green fluorescent protein–tagged murine leukemia virus pseudotype in lieu of pathogenic rabies virus, reducing the need for specialized reagents for antigen detection and enabling use in low-containment laboratories. We further used statistical analysis to generate rapid, quantitative predictions of the probability and titer of rabies virus neutralizing antibodies from microscopic imaging of neutralization outcomes. Using 47 serum samples from domestic dogs with neutralizing antibody titers estimated using the fluorescent antibody virus neutralization test (FAVN), pmRFFIT showed moderate sensitivity (78.79%) and high specificity (84.62%). Despite small conflicts, titer predictions were correlated across tests repeated on different dates both for dog samples ($r = 0.93$), and for a second dataset of sera from wild common vampire bats ($r = 0.72$, $N = 41$), indicating repeatability. Our test uses a starting volume of 3.5 µL of serum, estimates titers from a single dilution of serum rather than requiring multiple dilutions and end point titration, and may be adapted to target neutralizing antibodies against alternative lyssavirus species. The pmRFFIT enables high-throughput detection of rabies virus neutralizing antibodies in low-biocontainment settings and is suited to studies in wild or captive animals where large serum volumes cannot be obtained.

Key words: biologic assay, generalized linear mixed models, immunofluorescence, vampire bat

Introduction

The last few decades have seen a surge in newly emerging human viruses that originate from wildlife (Cunningham, Daszak, & Wood, 2017; Daszak, Cunningham, & Hyatt, 2000; Goodin, Jonsson, Allen, & Owen, 2018). Key examples include Nipah virus (Gurley et al., 2017), Seoul virus (Kerins et al., 2018), 2009 H1N1 (Mena et al., 2016), and the recent SARS-CoV-2 (Zhou et al., 2020). Understanding the epidemiological dynamics of such viruses within their natural host populations is a fundamental component to discerning the spatiotemporal dynamics of past outbreaks and anticipating future emergence (Cunningham et al., 2017; Plowright et al., 2016). Investigating the dynamics of zoonotic

viruses within wildlife presents multiple challenges such as limited sample sizes, biased sampling and multiple diagnostic tests that are difficult to compare. Moreover, viruses themselves may be undetectable at the moment of sampling when infections periods are short or virus shedding is intermittent (Becker, Crowley, Washburne, & Plowright, 2019; Gilbert et al., 2013; Plowright, Becker, McCallum, & Manlove, 2019). Consequently, key parameters needed to inform population-level disease dynamics (e.g. incidence, infection and incubation periods) are difficult or impossible to measure directly (Borremans, Hens, Beutels, Leirs, & Reijniers, 2016). Serological tests offer a powerful alternative to approaches that rely on pathogen detection (Gilbert et al., 2013). Since pathogen-specific antibodies generally persist longer than the pathogen itself, serological data can inform individual exposure histories, population seroprevalence, and in some cases, help approximate the force of infection (Borremans et al., 2016; Gamble, Garnier, Chambert, Gimenez, & Boulinier, 2020; Gilbert et al., 2013; Metcalf et al., 2016). However, applying serology to longitudinal studies of wildlife presents distinct challenges from tests used in clinical diagnostic settings, where accuracy takes precedence over scalability. In particular, serological tests for studies of wildlife should be amenable to the small volumes of serum that often characterize collections from small-bodied hosts (e.g. rodents, bats, birds), scalable to large numbers of samples required for such studies, and possible to implement in low-biocontainment laboratories.

Rabies lyssavirus (RV; Genus *Lyssavirus*, Family *Rhabdoviridae*) is a zoonotic virus transmitted in saliva by the bite of infected mammals (mainly Carnivora and Chiroptera) (Rupprecht, Kuzmin, & Meslin, 2017). RV constitutes a substantial global health problem that is responsible for over 59,000 human fatalities annually, mostly attributable to domestic dogs (WHO, 2017). In Latin America, vampire bat RV causes more cases of human and domestic animal rabies mortality than RV transmitted by dogs; similarly, bats are among the most frequently diagnosed sources of human rabies exposure in the USA (Ma et al., 2020; Schneider et al., 2009). Bat lyssaviruses exemplify the challenges of studying zoonoses using pathogen detection and the potential advantages of serological inference (Turmelle et al., 2010). Active infections are rarely detected due to low population-level incidence and the short infectious period that precedes death (Jackson et al., 2008; Turmelle et al., 2010). However, abortive infections (i.e. bats that are exposed to RV but do not become infectious) are routinely observed through antibody presence in apparently healthy bats (Constantine, Tierkel, Kleckner, & Hawkins, 1968; Jackson et al., 2008; Obregón-Morales et al., 2017; Steece & Altenbach, 1989). As such, antibody detection is commonly used for ecological and epidemiological studies of bat rabies (Blackwood, Streicker, Altizer, & Rohani, 2013; Costa et al., 2013; de Thoisy et al., 2016; George et al., 2011; Horton et al., 2020; Streicker et al., 2012; Wright et al., 2010).

Existing tests to detect RV antibodies include enzyme-linked immunosorbent assays (ELISAs) (Barton & Campbell, 1988; Cliquet et al., 2004; Wasniewski & Cliquet, 2012) and virus neutralization tests (Cliquet, Aubert, & Sagné, 1998; Smith, Yager, & Baer, 1973). ELISAs are a rapid and effective method for testing large numbers of samples, but they do not detect virus neutralization (Ma, Niezgoda, Blanton, Recuenco, & Rupprecht, 2012; Welch, Anderson, & Litwin, 2009). Moreover, since ELISAs ideally require a secondary antibody specific to the host species of the original sample (Reynes et al., 2004), which is often non-existent, they are not widely used in wildlife rabies surveillance. Tests detecting virus neutralizing antibodies (VNA) are more commonly adopted (De Benedictis, Mancin, Cattoli, Capua, & Terrregino, 2012; Irie & Kawai, 2018; Moore & Hanlon, 2010). Specifically, the fluorescent antibody virus neutralization test (FAVN) and the rapid fluorescent focus inhibition test (RFFIT) are considered the gold standard for measuring vaccination response by the World Health

85 Organization (WHO) (Briggs et al., 1998; De Benedictis et al., 2012). Both the FAVN and RFFIT have
86 been modified to facilitate use with wildlife samples. For instance, Kuzmin et al. (2008) modified the
87 RFFIT into a micro-neutralization test using 4-well Teflon coated slides instead of 8-well chamber
88 slides. This produced a sensitive and specific test that only required 3.5 µl of serum (regular RFFIT and
89 FAVN require ~50 µL of serum) (Kuzmin et al., 2008). Several other laboratories have introduced the
90 modification of pseudotype viruses (i.e. non-rabies viruses engineered to express heterologous
91 envelope glycoproteins) to quantify VNA titers without highly pathogenic live lyssaviruses (Temperton,
92 Wright, & Scott, 2015), which in most countries involves biosafety level (BSL)-3 laboratories to produce
93 concentrated virus stocks (WHO, 2018). Pseudotyped viruses based on vesicular stomatitis virus
94 (Moeschler, Locher, Conzelmann, Krämer, & Zimmer, 2016), human immunodeficiency virus, and
95 murine leukemia virus (MLV) (Wright et al., 2008) tend to be highly sensitive and specific relative to
96 live lyssavirus counterparts. Viral pseudotypes have also been modified to incorporate molecular
97 biomarkers such as firefly luciferase, renilla luciferase, or green fluorescent protein (GFP), eliminating
98 the need to fix and stain cells with a fluorescein isothiocyanate (FITC) conjugated rabies antibody,
99 which reduces test cost (Bentley, Mather, & Temperton, 2015; Mather et al., 2013; Moeschler et al.,
100 2016; Moore & Hanlon, 2010; Wright et al., 2008).

101 Despite these improvements, existing variations of RV neutralization tests have several limitations.
102 First, all tests require serial dilutions of serum samples, each of which requires manual microscope
103 scoring that increases personnel time and risks of error (Moeschler et al., 2016; Péharpré et al., 1999).
104 Second, testing is commonly split into screening and end-point titration phases, requiring tests to be
105 run in duplicate or sequentially. Third, pseudotypes have not yet been integrated into micro-
106 neutralization tests, implying that tests to detect VNAs against most lyssaviruses in their natural bat
107 reservoirs can only be carried out in BSL-3 or higher facilities. The growing demand to study wildlife
108 on large spatial and temporal scales would benefit from minimizing these logistical constraints (Becker
109 et al., 2019).

110 The ideal serological diagnostic would be a scalable virus neutralization test that requires low-volume
111 samples and can be carried out in any standard microbiology/cell culture laboratory. Here, the micro-
112 neutralization RFFIT is adapted to use an MLV-based viral pseudotype bearing the RV glycoprotein and
113 carrying a GFP marker gene. Further, we introduce a novel quantitative approach that combines digital
114 image analysis of infected cells with statistical analysis, which allows us to estimate rabies virus
115 neutralizing antibody (RVNA) titers without multiple serum dilutions or rounds of testing. Our
116 approach relies on the expectation of a negative relationship between RVNA concentrations in serum
117 and the number of cells that become infected upon viral challenge in the presence of that serum.
118 Defining that relationship quantitatively using known titers of standard rabies immune globulin (SRIG)
119 allows predicting the presence and, when appropriate, titers of RVNAs in test sera. Our approach,
120 which we refer to as a pseudotype micro-neutralization RFFIT (hereafter, pmRFFIT, **Figure 1**) provides
121 a safe, low-cost alternative to standard RV neutralization tests that is suitable for large-scale,
122 population-level studies or laboratory studies where small animals are longitudinally sampled.

123 **Methods**

124 *Laboratory work*

125 Cell lines. For all the neutralization tests, mouse neuroblastoma cells, N2A (Neuro-2A, ATCC®
126 CCL131™), were cultured in Minimum Essential Media (MEM) supplemented with 10% fetal bovine

127 serum (FBS), 1% 100x non-essential amino acid (NEAA), 1% 200mM L-glutamine and 1% antibiotic-
128 antimycotic. For viral pseudotype production, human embryonic kidney 293T cells (293T, ATCC® CRL-
129 3216™), were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS,
130 1% 200mM L-glutamine, 100 units/mL Penicillin, 1 µg/ml Streptomycin and 400 µg/mL G418
131 (Geneticin) antibiotic.

132 Pseudotype production. MLV pseudotypes expressing the challenge virus standard-11 (CVS-11) rabies
133 virus glycoprotein (MLV(RG)) were generated by co-transfection of pCMVi (MLV gag-pol expression
134 vector), pCNCG (MLV viral origin and GFP reporter gene), and pI.18-CVS-11 (plasmid encoding the CVS-
135 11 RG) (Bock, Bishop, Towers, & Stoye, 2000; Towers et al., 2000; Wright et al., 2008). Plasmids were
136 transfected using polyethylenimine (Polysciences, Inc.) into 293T cells at a ratio of 1:1.5:1 of
137 pCMVi/pCNCG/pI.18-CVS-11. After 72 hours, the supernatant containing the viral pseudotypes was
138 harvested, aliquoted and stored at -80°C until further use. Virus concentration was determined in N2A
139 cells by calculating the TCID50 (50% tissue culture infective dose) using the end point method and
140 using the Spearman-Kärber formula (Condit, 2013; Hierholzer & Killington, 1996).

141 Neutralization test. A two-dilution micro-neutralization test was established following Kuzmin et al.
142 (2008); however, in place of 4-well Teflon-coated slides, 6-well Teflon-coated slides (Tekdon Inc.) were
143 used, enabling 3 samples to be analyzed per slide. Each serum sample (starting volume of 3.5 µL), was
144 screened at a 1:10 and 1:25 dilution (a total of 2 wells per sample). Serum dilutions were inoculated
145 with 12.5 µl of MLV(RG) (viral input: 300-400 $TCID_{50}$) and incubated in humidified square petri dishes
146 at 37°C, 5% CO_2 for 90 min. After incubation, 25 µl of N2A cells ($2x10^6$ cells/ml) were added to the
147 serum-virus mixture and slides were incubated at 37°C, 5% CO_2 for 66 to 72 h in the humidified square
148 petri dishes. This extended incubation period (compared to the FAVN (48 h) or the RFFIT (20 to 24 h))
149 was required for the MLV(RG) to generate sufficient GFP expression for later microscopy. Since our
150 pseudotype virus used GFP to indicate viral entry into cells, neither acetone fixation nor staining with
151 anti-rabies monoclonal globulin were required. Every neutralization test included three internal
152 controls: 1) a cell-only control where no virus or serum was added to the well; 2) a virus control,
153 comprising back titrations of the MLV(RG) with: a) the original concentration of the virus (300-400
154 $TCID_{50}$), b) a 1:10 dilution of the virus, and c) a 1:100 dilution of the virus; and 3) a negative control for
155 neutralization, where we added 300-400 $TCID_{50}$ of MLV(RG) to two wells without any serum. A
156 standard curve was generated with each neutralization test describing the expected number of
157 infected cells versus titer concentration. For this, the same protocol was used with six different titer
158 concentrations of SRIG in lieu of serum samples: 0.01, 0.025, 0.05, 0.1, 0.15 and 0.2 IU/mL
159 (reconstituted at 30 IU/mL in 1.0 mL of nuclease-free water, 2nd WHO International Standard, Human;
160 NIBSC, UK) (**Figure 1** A).

161 *Imaging and data processing*

162 After incubation, slides were photographed at 4x magnification under a fluorescence microscope
163 (EVOS FL Cell Imaging System). Wells were divided into five equally sized fields (four corners and
164 center) and photographed clockwise from the top left corner, such that the fifth photograph was
165 always the center field. Each photograph was processed through the program ImageJ (version 1.52k
166 (Rasband, 2018)) using the Autolocal Threshold Phansalkar plugin (size radius 15) (part of the Fiji
167 distribution (Schindelin et al., 2012)), where each image was transformed into a binary image (every
168 pixel stored as a single bit). This indicated the presence (white) and absence (black in the background)
169 of GFP fluorescence. Next, the command "Analyze Particles" was used to count the total number of

170 fluorescent cells per field (i.e. infected cells). This command grouped and counted the white
171 neighboring pixels with a predetermined size area and circularity to be a single cell (size area: 5-50
172 circularity: 0.80-1.0), so counts corresponded to the number of infected cells. Cell count outputs were
173 converted into a standardized spreadsheet using a Python version 3.7.2 script (Python Core Team,
174 2019) (script available in supplementary materials). At the end of the image processing step, each
175 serum sample was described by 10 data points consisting of the number of the fluorescent cells in
176 each of 5 fields (photographs) in the 1:10 and 1:25 dilutions (**Figure 1** B).

177 *Statistical analysis*

178 All statistical analyses were executed in *R* (R Core Team, 2018). To predict the probability of RVNA
179 presence, a generalized linear mixed model (GLMM) with a binomial distribution was fit to the binary
180 outcome of the cell counts from the SRIG concentration series, where titers $\leq$ 0.1 IU/mL were
181 considered RVNA negative and $>$ 0.1 IU/mL were considered RVNA positive. Other serology studies in
182 wildlife have used similar thresholds to detect RVNA (Araujo et al., 2014; Azevedo de Paula Antunes
183 et al., 2017; Campos et al., 2019; Silva et al., 2010). To next predict RVNA titers, a GLMM with a log-
184 normal distribution was fit to the infected cell counts across the SRIG concentration series. For both
185 the binomial and log-normal models, a model was constructed including all data (i.e. from 1:10 and
186 1:25 dilutions) where two fixed effects were considered: 1) the count of virus-infected N2A cells
187 (scaled to improve model convergence) and 2) the serum dilution level (two factors: 1:10 and 1:25
188 dilution). Random slope and intercept terms were also considered for the date the test was run ("test
189 date") to account for observed variation in the relationships between SRIG titers and infected cell
190 counts across dates (**Figure 2**) and for the field number (1 to 5) within each microscope well ("field")
191 to account for variation in cell counts between fields (the middle field, field 5, had more agglomerated
192 cells in particular). To evaluate whether a simpler, single dilution test produced comparable results,
193 the full dataset was then subset to the 1:10 dilution only. The binomial and log-normal models fit to
194 this data subset included only the fixed effect of the virus-infected N2A cell counts, but the random
195 effects were identical to those explained above (i.e. test date and field). Models were fit using the
196 *'lme4'* package (Bates et al., 2015). The '*predict'* function was used to generate the predicted
197 probability that a SRIG concentration or serum sample was seropositive (binomial model) and its
198 corresponding RVNA titer (log-normal model). Predictions per field were averaged to obtain results
199 per sample (**Figure 1** C).

200 <u>Model selection.</u> To select the best-performing models, a top-down model selection strategy was
201 implemented (Zuur, Ieno, Walker, Saveliev, & Smith, 2009). First, we identified the optimal random
202 effects by comparing models with alternative random effect structures, but the same fixed effects,
203 using the Akaike Information Criterion corrected for small sample size (AICc) calculated using the
204 *'MuMIn'* package (Bartoń, 2013). A lower AICc with a difference of at least two values (ΔAICc > 2) was
205 considered as evidence of improved model fit. Next, keeping the chosen random effects, models with
206 the two different datasets were evaluated (simpler model: 1:10 dataset, and more complex model:
207 1:10 and 1:25 dataset) (**Table 1**). Since models fit to different datasets cannot be compared through
208 AICc, the simpler and more complex models were compared using predictions for SRIG (sera with
209 known titers). For the binomial models, the sensitivity and specificity with a threshold of > 0.1 IU/mL
210 for seropositivity were used. For the log-normal models, the Spearman correlation coefficients of
211 predicted and known SRIG titers were compared.

212 *Test repeatability and external validation*

213 Dog (N = 47) and bat (N = 41) sera were used to assess pmRFFIT repeatability. Dog sera had previously
214 been tested using FAVN at the Animal Health and Veterinary Laboratories Agency, Weybridge (Wright
215 et al., 2009). Bat sera were collected from common vampire bats (*Desmodus rotundus*) as part of an
216 ongoing field study in Peru (collection permit: 0142-2015 SERFOR/DGGSPFFS; exportation permit:
217 003327-SERFOR; SI 1).

218 To quantify repeatability, dog and bat sera were processed using the pmRFFIT as described above on
219 two separate dates. For the binomial model, repeatability was measured as the proportion of samples
220 with the same predicted serological status on both test dates. For the log-normal model, repeatability
221 was calculated as the Spearman correlation coefficient of predicted RVNA titers from different test
222 dates.

223 The sensitivity and specificity of the pmRFFIT were calculated relative to the FAVN using binomial
224 predictions from dog sera. Sera with FAVN titers > 0.1 IU/mL were classified as positive and ≤ 0.1IU/mL
225 as negative. Only data from the first test date were used under the assumption that future applications
226 would preferably use a single test. To compare pmRFFIT accuracy in different FAVN titer ranges, the
227 RVNA values obtained through FAVN were categorized through the '*cut*' function in R. The number of
228 categories was obtained by dividing the range of the RVNA titer values (maximum minus minimum
229 value obtained through FAVN) by the optimal category width, calculated with the Freedman-Diaconis
230 rule (Freedman & Diaconis, 1981). Later, to evaluate the accuracy of the binomial prediction in the
231 lowest FAVN titers, the categorization procedure was repeated but only using the lowest resulting
232 category from the previous step. A Receiver Operating Characteristic (ROC) curve analysis was used to
233 explore the accuracy of the log-normal predictions across the range of evaluated titers. FAVN titers
234 with a threshold of > 0.1 IU/mL (positives) were used as the benchmark reference.

235 **Results**

236 To understand the variability of the pmRFFIT, replicate SRIG titer concentration curves were produced
237 on 6 different dates between 30/05/2019 and 27/06/2019 (hereafter "Test 1" through "Test 6"). As
238 expected, the number of infected cells declined at higher SRIG titers in all replicates; however, the
239 shape of the antibody decay curve varied across test dates (**Figure 2**). At the 0.1 IU/mL SRIG
240 concentration, infected cell counts were more dispersed in the 1:25 dilution than in the 1:10 dilution,
241 as indicated by higher interquartile range (IQR) within each of the 6 test dates. Across all the SRIG
242 concentrations in all test dates (N = 36), 77.78% of the count comparisons were less dispersed in the
243 1:10 dilution suggesting this dilution could be more precise for downstream statistical analysis (SI 2).

244 *Prediction of seropositivity using binomial GLMM*

245 Binomial GLMMs accurately predicted seropositive and seronegative SRIG concentrations (**Figure 3**).
246 The best random effects for the binomial model included a random slope and intercept for test date
247 (**Table 1**). The models built with the 1:10 dilution data only ("one-dilution model") and from both the
248 1:10 and 1:25 dilution data ("two-dilution model") had equivalent specificity (100%), but the one-
249 dilution model was more sensitive (100% versus 58.33%, **Figure 3** A, B). Furthermore, the two-dilution
250 binomial model failed to correctly predict the seropositive controls on 4 out of the 6 test dates,
251 confirming improved performance of the one-dilution model (**Figure 3** B).

252 *Prediction of titer values using log-normal GLMM*

253 The log-normal GLMMs gave repeatable predictions of RVNA titers from the datasets generated
254 through our protocol across test dates (**Figure 3** C). The best log-normal model included a random
255 intercept and slope for test date. Although the most complex model had the lowest AICc, the simpler
256 model (without the random intercept of field) had a ΔAICc < 2 (**Table 1**). Observed and predicted SRIG
257 titers were highly correlated for both the one and two-dilution models (r = 0.95, **Figure 3** C). When
258 comparing test dates (i.e. one-to-one comparison between correlations of the one-dilution and the
259 two-dilution model from the same test dates), the correlation coefficients were similar, suggesting the
260 simpler one-dilution model is sufficient for titer prediction (**Figure 3** D).

261 *Repeatability and external validation with animal sera*

262 Among all the animal serum samples (N = 88), 95.45% were consistently predicted as seropositive or
263 seronegative (92.68% for bats and 97.87% for dogs). Bat samples (N = 41) had a repeatability of 66.7%
264 for positive samples (6/9 positive samples were positive in the second round) and 100% for negative
265 samples (N = 32). Dog samples (N = 47) had 100% repeatability for positive samples (N = 28) and
266 94.74% for negative samples (18/19 negative samples remained negative in the second round) (**Figure**
267 **4** A, SI 3). Predicted titers between test repetitions were correlated for both sera (both r = 0.81, bat r
268 = 0.72, dog r = 0.93, **Figure 4** B).

269 Relative to FAVN, pmRFFIT predictions using the binomial model on dog sera showed overall moderate
270 accuracy (80.43% of samples in agreement with FAVN; sensitivity = 78.79%; specificity = 84.62%).
271 Accuracy was higher for the dog samples categorized as positive according to the binomial prediction
272 (N = 28), where only 2 samples failed in the pmRFFIT prediction compared to the FAVN. For dog
273 samples categorized as negative by the binomial pmRFFIT (N = 9), 7 samples were contradictory. These
274 misclassified samples were consistently predicted as negative in the pmRFFIT, and titers displayed a
275 strong correlation between tests (r = 0.93, SI 4). The binomial model was most accurate for samples
276 with high RVNA titers (> 1.04 IU/mL, N = 13; 100% predicted to be seropositive; **Figure 5** A). At lower
277 FAVN titers (< 1.04 IU/mL, N = 33), 72.73% of samples were predicted correctly by the pmRFFIT. To
278 evaluate the accuracy of the binomial prediction in this lower range, the titer categorization process
279 was repeated using the subset of data with FAVN titers ≤ 0.871 IU/mL (N = 33). Among the higher titer
280 samples in this particular set (> 0.27 to ≤ 0.871 IU/mL, N = 11), 90.91% were accurately predicted as
281 seropositive (**Figure 5** B). From the samples with titers ≤ 0.27 IU/mL, only 63.64% were accurately
282 predicted (N = 22, **Figure 5** B). Accuracy rose to 83.33% in the lowest FAVN titer range (≤ 0.07 IU/mL,
283 N = 12). The ROC curve showed that threshold values between 0.099 and 0.166 IU/mL maximized
284 sensitivity and specificity.

285 **Discussion**

286 The most commonly applied serological tests to detect RVNA titers challenge a range of serial dilutions
287 of serum with infectious RV. This process is labor-intensive and requires laboratory capacity to grow
288 large quantities of pathogenic RV. Here, we provide an alternative serological framework that uses a
289 combination of digital image analysis and statistical analysis to estimate the presence and titer of
290 RVNA from a single dilution using only 3.5 µL of serum.

291 The pmRFFIT differs from other lyssavirus neutralization tests in several key aspects. It uses an
292 MLV(RG) pseudotype rather than pathogenic RV, allowing the pmRFFIT to be performed in any low-
293 containment laboratory with appropriate cell culture and microscopy facilities. The addition of GFP
294 expression is significant, since it removes the need for FITC-conjugated antibody (reducing reagent

295 costs) and the fixation and staining steps used in traditional RFFIT or FAVN. One potential drawback
296 of using GFP expression to measure infectivity is the prolonged neutralization period (66 h versus 24
297 h RFFIT and 48 h FAVN) required to gain sufficient fluorescence for image processing (Aubert, 1992;
298 Smith et al., 1973). Longer neutralization requirements (60 h) were also required in a FAVN
299 modification using a GFP expressing recombinant CVS-11-eGFP but did not alter results relative to the
300 test run with CVS-11 (Xue et al., 2014). Fortunately, extended incubations are unlikely to alter
301 neutralization outcomes since MLV(RG) pseudotype is replication incompetent, preventing infection
302 of additional cells during the incubation (Temperton et al., 2015). The pmRFFIT also uses an imaging
303 pipeline that combines systematic photography of microscope fields with automated digital image
304 processing to count infected cells. Microscopy in neutralization tests is time consuming and presents
305 challenges for interlaboratory comparisons due to multiple sources of variation, especially those that
306 affect the manual readout (e.g. laboratory user, manual pipetting, uneven cell monolayer) (Briggs et
307 al., 1998; Cliquet et al., 1998; Eschbaumer et al., 2014; Péharpré et al., 1999; Timiryasova et al., 2019).
308 The pmRFFIT standardized approach minimizes these sources of error while potentially reducing
309 microscope operator time. Moreover, the imaging process generates traceable and permanent
310 electronic records of the raw data, eliminating the need to manually digitize records of field counts.
311 Several investigators have previously incorporated image processing into RV neutralization tests. For
312 example, Pérhapré et al. (1999) modified the FAVN and RFFIT readout by using an automated image
313 analysis, however one important drawback was the high cost of the equipment (a motorized stage
314 and the software required for this). Streicker et al. (2012) also used photography and image processing
315 to count pixels using a microRFFIT but did not make full use of the quantitative nature of imaging data
316 to obtain RVNA titers and used pathogenic RV rather than a viral pseudotype. A final distinction is that
317 instead of scoring microscope field or wells as virus positive or negative, the pmRFFIT predicts
318 serological status and RVNA titer from infected cell counts in a single serum dilution using statistical
319 modelling. The efficacy of this approach highlights the value of historically underutilized quantitative
320 data on cellular infectivity for lyssavirus serology.

321 Model selection indicated substantial day-to-day variation in the SRIG dilution series. This was
322 unsurprising, since virus neutralization tests are biologically dynamic systems that can be influenced
323 by many factors (e.g. variability in the humidity of the incubator, technical manipulation, light
324 condition of the microscope, variability in GFP expression in the cells) (Briggs et al., 1998; Hammami
325 et al., 1999; Kostense et al., 2012). Since our statistical approach handles this variability through the
326 random effect of test date, the pmRFFIT is best suited for large numbers of serum samples that require
327 testing to be carried out across multiple batches. However, performance is only marginally reduced
328 when running single models for each test date, implying the pmRFFIT may still be useful when fewer
329 samples are available for testing (see SI 5, 6). Surprisingly, fitting the GLMMs to data from a single 1:10
330 dilution of SRIG predicted both seropositivity and RVNA titer more accurately than models fit to both
331 the 1:10 and 1:25 dilutions. The reduced performance of the two-dilution model reflected higher
332 variability in the 1:25 dilution compared to the 1:10 dilution, as evidenced by greater IQR values (SI
333 2). Ultimately, this variability likely reflects both higher stochasticity in infected cell counts at lower
334 serum concentrations and pipetting error. Regardless, the ability to detect low titers (< 0.2 IU/mL)
335 with just one dilution and without the need to conduct separate tests for screening and titration is
336 advantageous since manual effort and materials are reduced.

337 Cell-based experiments are expected be more variable than other serological diagnostics, and often
338 the allowed variation for test precision is up to 30% (Kostense et al., 2012; Timiryasova et al., 2019).

339 For example, Kostense et al. (2012) evaluated RFFIT repeatability by testing 3 different types of
340 immunoglobulins (HRIG, CL184 and SRIG), and intra-test variation was 26%, 18% and 25% respectively.
341 Similarly, Timiryasova et al. (2019) validated a RFFIT protocol with 15.7% variation among intra-test
342 repetitions. In comparison, our test showed higher repeatability for serum samples from dogs and
343 similar repeatability for serum samples from bats. Positive prediction for bat samples had a lower
344 repetition, but most of the conflicts occurred with titers near the threshold defining seropositivity (2/3
345 samples, **Figure 4** B, SI 3) challenging a repeatable prediction between tests. The higher repeatability
346 in dog sera relative to bat sera is likely related to the presence of noticeably higher neutralizing
347 antibody titers in the dog sera, with 26/47 dogs (most of which had been vaccinated), compared to
348 5/41 bat samples having high predicted titers (≥ 0.15 IU/mL). Consistent with our findings, Kostenze
349 et al. (2012) also observed that antibody concentration curves had an initial and evident bend in the
350 titer concentration at 0.2 IU/mL, but the ability to observe slope changes in the lowest concentrations
351 of serum (< 0.1 IU/ml) was challenging. Overall, the pmRFFIT was most precise in discriminating strong
352 positives from strong negatives, where the highest and lowest titers were constantly predicted as
353 such. In consideration of these points, standard serological methods might have limitations measuring
354 low RVNA titers (< 0.5 IU/mL) while the pmRFFIT grants a highly repeatable approach for these titer
355 values.

356 Using the external validation set of dog samples with known RVNA titers, the overall accuracy of the
357 pmRFFIT was moderately high. Discrepancies occurred in samples with FAVN titers ranging from 0.07
358 to 0.29 IU/mL but were not observed for samples with higher titers (N = 23). In the previous analysis
359 of these samples, sera that did not reach the 0.5 IU/mL threshold were considered negative and were
360 not re-tested (Wright et al., 2009). We therefore cannot disregard the possibility of inaccurate titer
361 assessment through FAVN, considering low-titer detection is even more challenging through classical
362 approaches and that titer prediction through FAVN tests can vary even for higher titers (i.e. samples
363 scoring 0.5 IU/mL can range from 0.38 to 0.66 IU/mL when repeated) (Liu, Zhang, Zhang, & Hu, 2012;
364 Wright et al., 2009). Although we did not carry out a formal validation for the bat samples, the
365 estimated seroprevalence was 0.21 (CI: 0.09, 0.35), consistent with several previous studies using the
366 RFFIT (de Thoisy et al., 2016; Steece & Altenbach, 1989; Streicker, Franka, Jackson, & Rupprecht, 2013;
367 Streicker et al., 2012). The ability of our approach to estimate VNA titers for all samples tested without
368 additional laboratory work provides an additional layer of information that can be further used to
369 gauge confidence in positive or negative predictions. For example, predicted titers abutting the
370 threshold for seropositivity might be viewed with caution.

371 Detection of low RVNA titers is important for epidemiological studies of rabies exposure (Gold,
372 Donnelly, Nouvellet, & Woodroffe, 2020). For example, bats sometimes produce low levels of RVNA
373 after viral exposures, and the antibody response can wane to undetectable levels within months after
374 the first exposure (Jackson et al., 2008; Turmelle et al., 2010). Indeed, the predicted titers among our
375 predicted seropositive bat samples were consistently less than < 0.12 IU/mL. For this reason, we
376 designed our test to detect low RVNA titers, defined as > 0.1 IU/mL, and the ROC analysis showed this
377 was within the optimal range of thresholds for sensitivity and specificity. Consequently, titers > 0.2
378 IU/mL, as might be required for studies concerned with protective levels following immunization,
379 cannot currently be estimated accurately. By pmRFFIT, such samples would be classified as
380 seropositive with a predicted titer of 0.2 IU/mL. If desired, predicting higher RVNA titers than the focus
381 of this study would require higher additional serum dilutions in conjunction with a greater range of
382 SRIG concentrations.

In summary, the pmRFFIT quantifies RVNA titers using a single, low-biocontainment test that requires only a single dilution of test sera. We recommend first employing the binomial model at a pre-determined threshold for seropositivity, then using the log-normal model to predict titers for the putatively positive samples (considered positive by the binomial model). If benchmark data are available, it would be possible to select the most sensitive and specific threshold after predicting titers for all available samples using the ROC curve analysis. Downstream analyses could use binomial and continuous data as needed for specific project objectives. The pmRFFIT could be extended to other lyssaviruses for similarly low-biocontainment testing of neutralizing antibodies. The ability to perform the test using the low-volume sera that are often available for studies of wild bats or longitudinal studies of captive bats is a particularly useful feature of our approach. Such studies are increasingly desirable to investigate the transmission dynamics of poorly understood bat viruses.

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Ethical statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. Bat capture and sampling methods were approved by the Research Ethics Committee of the University of Glasgow School of Medical Veterinary and Life Sciences (Ref 08a/15).

Conflicts of Interest Statement

The authors declare no conflict of interest.

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657

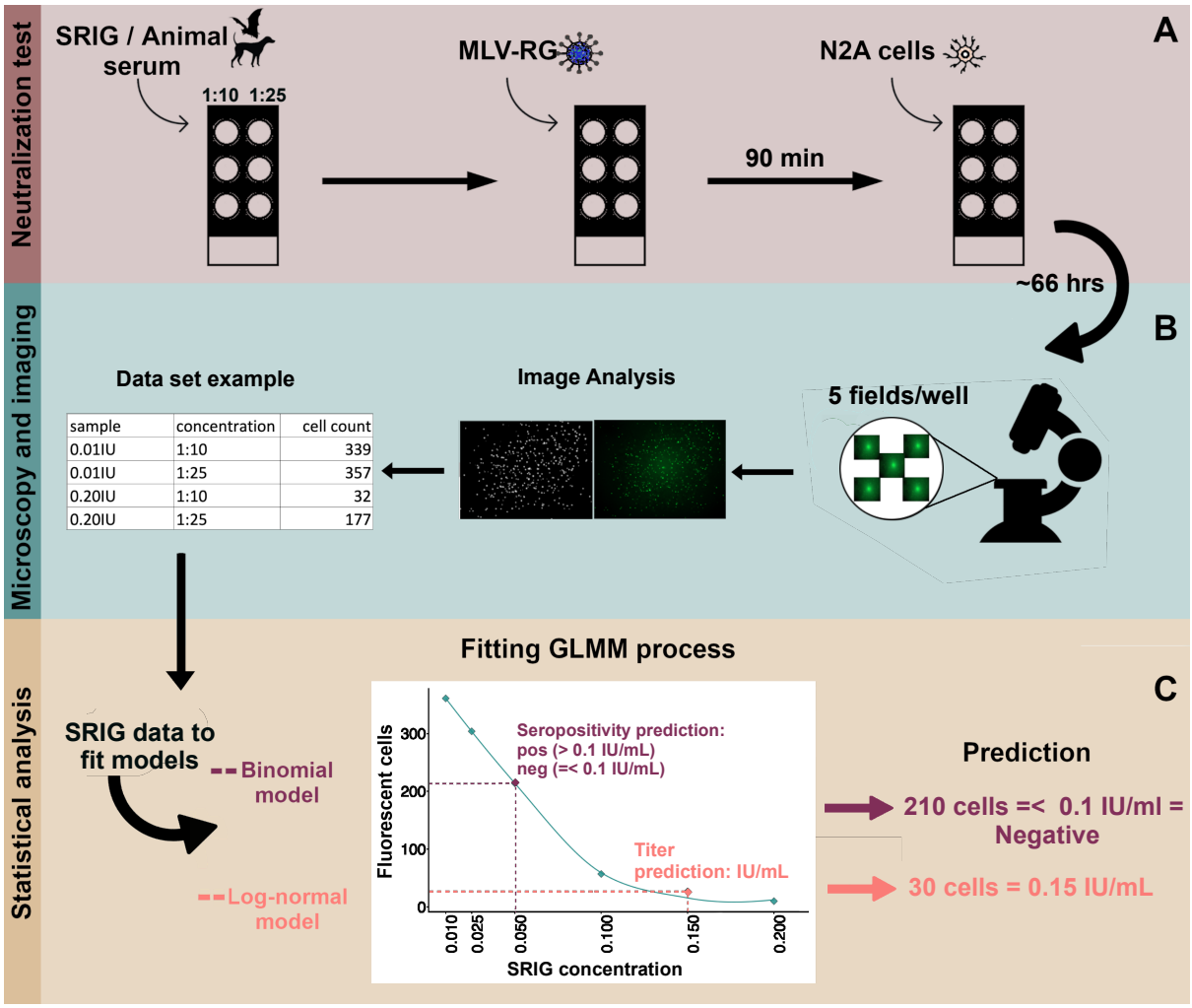
658 **Tables**

659 **Table 1.** Random effect structures investigated for the binomial (B) and log-normal (L) models, and
660 respective model fit measures: AICc, difference in AICc from best model (ΔAICc) and weight (W). The
661 fixed effects for all these models were identical and included the scaled count of the fluorescent
662 cells and the dilution effect

Model	Random effects	AICc	ΔAICc	*W*
B	*1. Test date as random intercept and random slope*	91.62	0.0	0.71
	2. Test date as random intercept and random slope + number of field as random intercept	93.49	1.87	0.28
	3. Test date as random intercept	102.32	10.70	0.0
	4. Test date as random intercept + number of field as random intercept	104.28	12.66	0.0
L	*1. Test date as random intercept and random slope + number of field as random intercept*	-1746.59	0.0	0.7
	2. Test date as random intercept and random slope	-1744.89	1.70	0.3
	3. Test date as random intercept + number of field as random intercept	-1637.32	109.27	0.0
	4. Test date as random intercept	-1637.30	109.29	0.0

663

664 **Figures**



665

666 **Figure 1.** Workflow of the pmRFFIT approach. A) Set up of the neutralization test using an MLV(RG)
667 pseudotype and 2 dilutions of either SRIG or animal serum. B) Microscopy phase and imaging to
668 perform the cell count of the fluorescent cells to construct a database to fit the statistical models. C)
669 Construction of the statistical models with two different types of prediction.

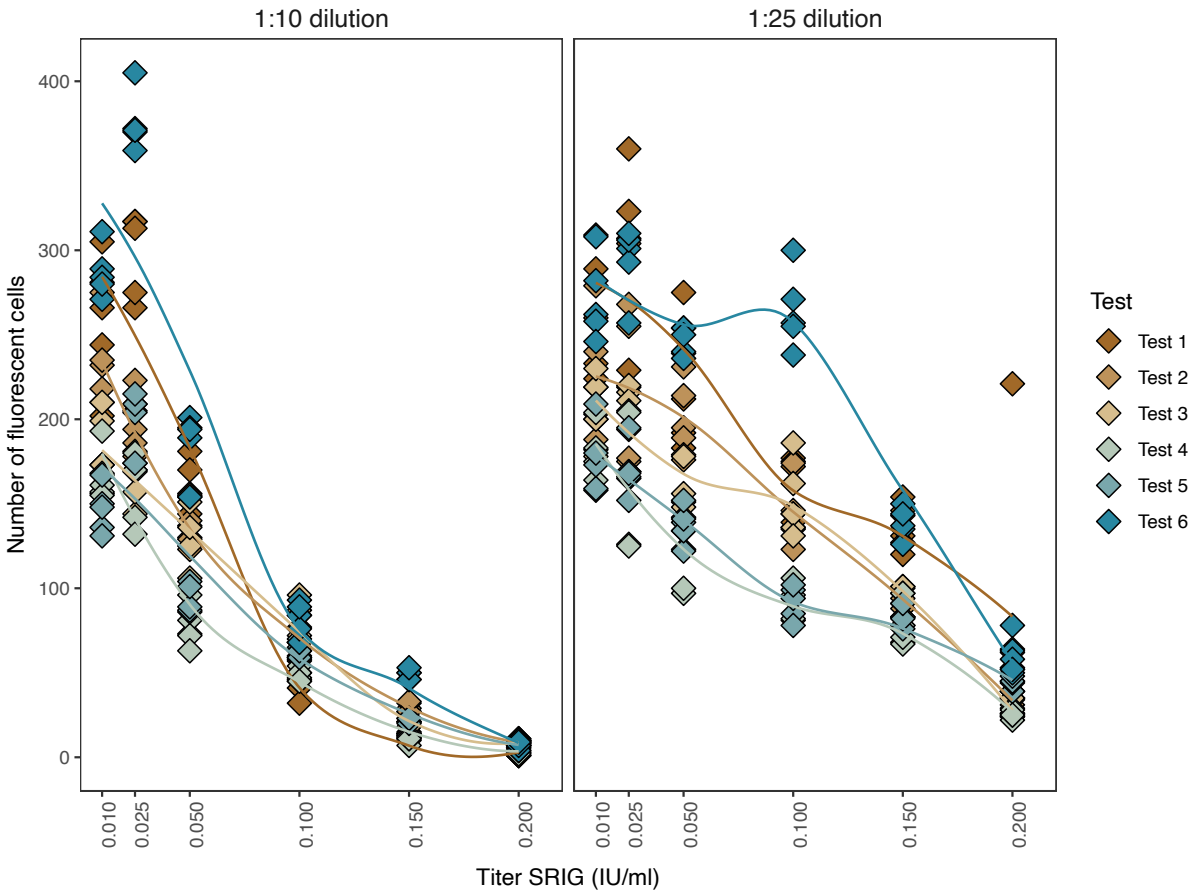
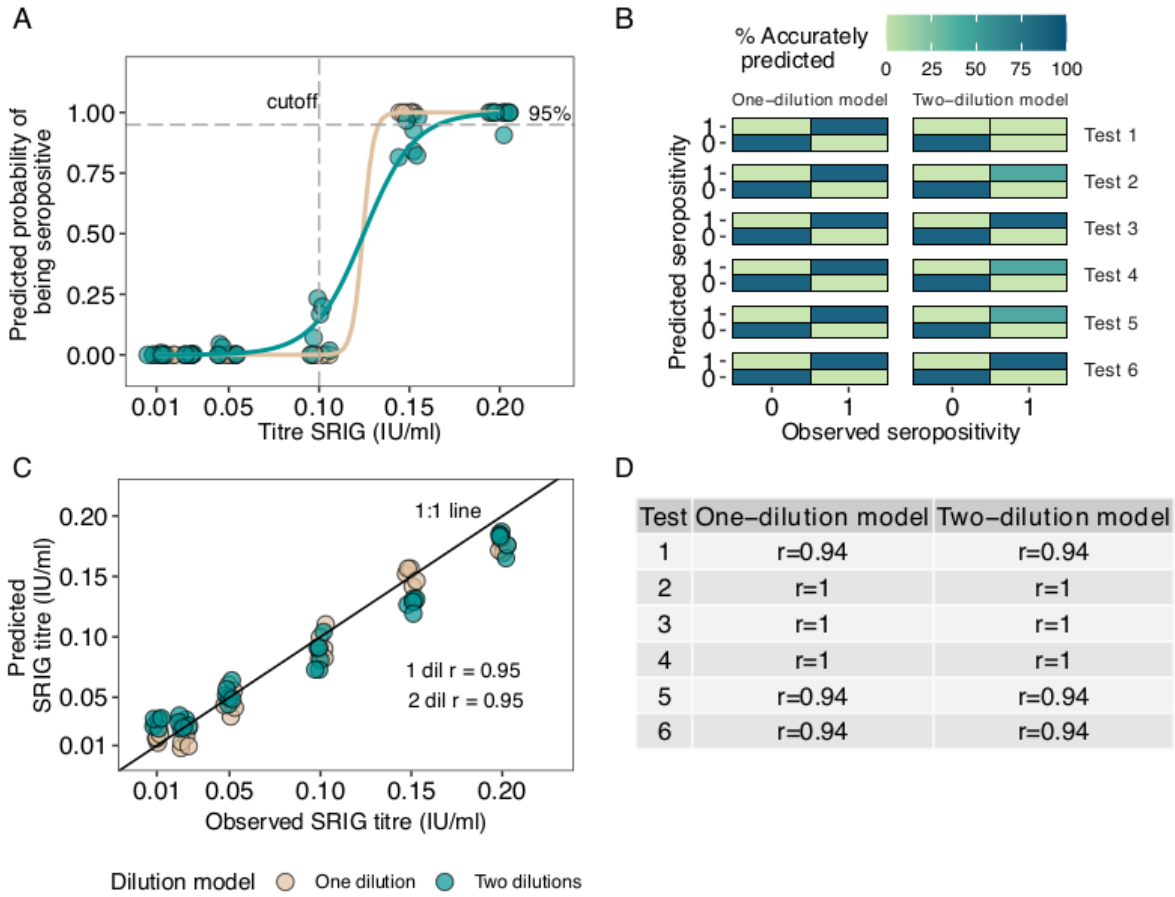


Figure 2. Raw counts of fluorescent cells for the SRIG concentration curves from 6 different pmRFFIT from different test dates and for each dilution. The dilutions were performed on the same date, with one well next to the other. Each color represents a different test date. The loess curves denote differences in the counts from test to test.



Test	One–dilution model	Two–dilution model
1	r=0.94	r=0.94
2	r=1	r=1
3	r=1	r=1
4	r=1	r=1
5	r=0.94	r=0.94
6	r=0.94	r=0.94

Figure 3. Results of the models fit to the SRIG control data. All graphs compare the one-dilution (1:10) and the two-dilution (1:10 and 1:25) models. A) Model fit of the titers of the SRIG concentration curve versus the predicted probability from the binomial model. The horizontal dash line represents the 95% probability and the vertical dash line represents the previously set threshold at 0.1 IU/mL. B) Sensitivity and specificity of the binomial prediction per test date. C) Correlation of the titers of the observed SRIG values versus the predicted values with the log-normal models. D) Comparison of the Spearman coefficients between the correlations of the observed titers versus the predicted titers per test date.

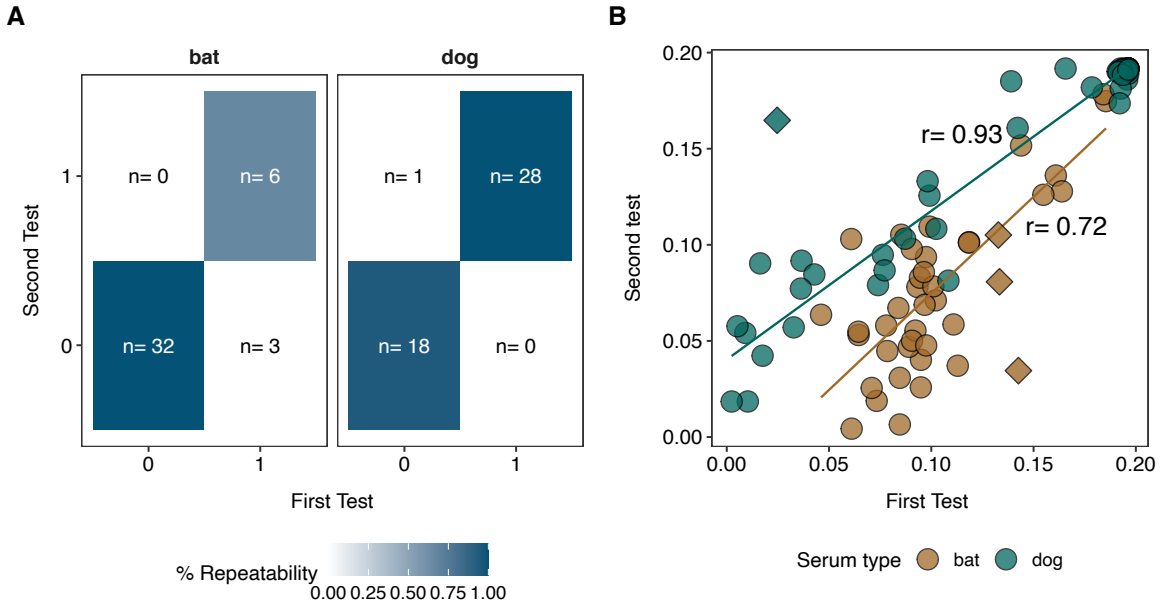


Figure 4. Repeatability of the predictions in bat (N = 41) and dog (N = 47) samples. A) Percentage of repeatability of the seropositive outcome of the bat and dog serum samples with the pmRFFIT test. B) Linear correlation of the predicted titers between the first and second test for dog serum and bat serum. Diamonds indicate samples that were contradictory in the binomial prediction.

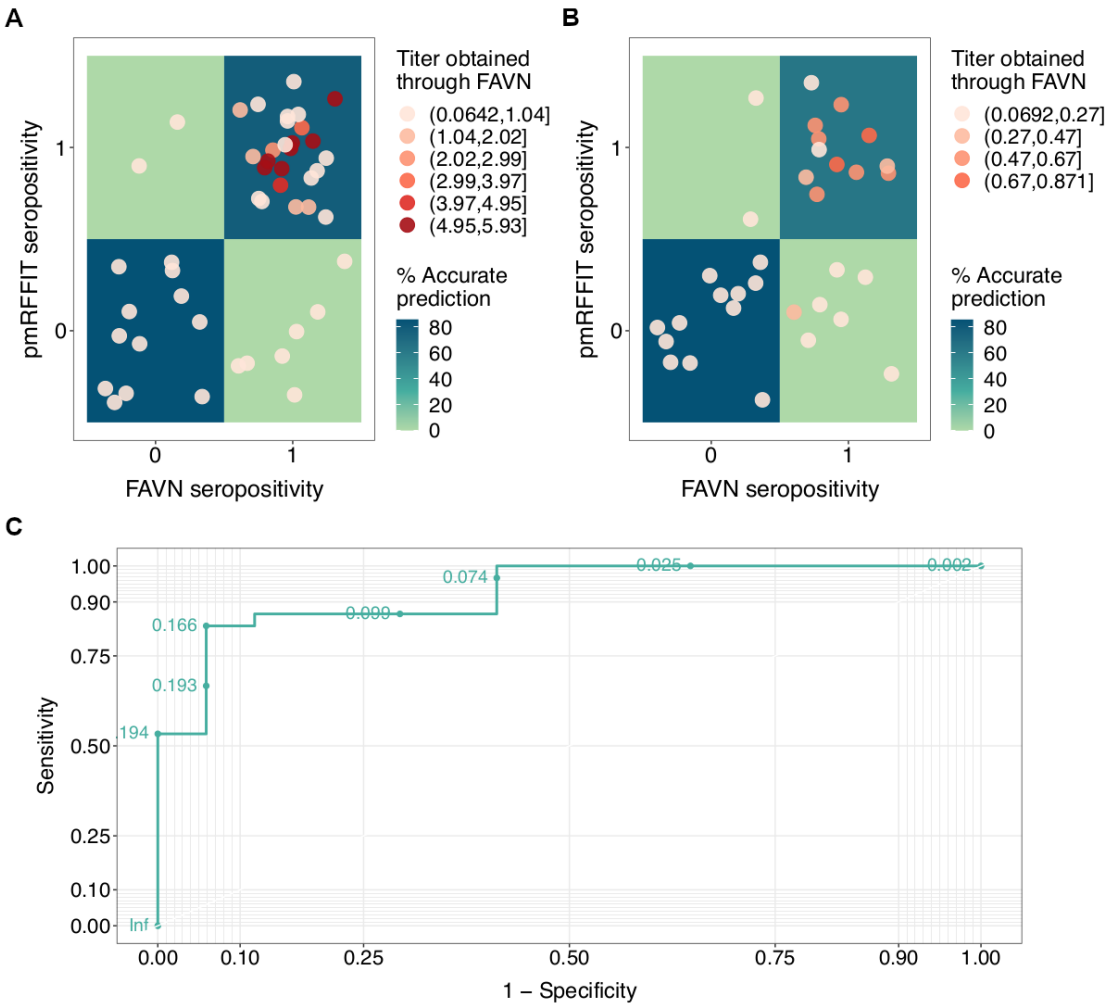


Figure 5. Validation of pmRFFIT with the FAVN on the dog serum samples. A) Seropositivity comparison for all FAVN titers: Sensitivity = 78.79%, Specificity = 85.71%, Overall accuracy = 80.85% (N = 47). B) Seropositive comparison of lower FAVN titers: Overall accuracy = 72.73% (N = 33). C) ROC curve of the lognormal titer prediction of the first test compared to the FAVN at threshold > 0.1 IU/mL for seropositivity. The continuous thresholds for the pmRFFIT are in green.