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1 **Development and Use of an Indirect Enzyme-linked Immunosorbent Assay for Detection of**
2 **Iridovirus Exposure in Gopher Tortoises (*Gopherus polyphemus*) and Eastern Box Turtles**
3 **(*Terrapene carolina carolina*)**

4
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24 **Abstract**

25 Iridoviruses, pathogens typically associated with fish and amphibians, have recently been shown
26 to cause acute respiratory disease in chelonians including box turtles, red-eared sliders, gopher
27 tortoises, and Burmese star tortoises. Case reports of natural infections in several chelonian
28 species in the United States have been reported, however the prevalence remains unknown in
29 susceptible populations of free-ranging chelonians. To determine the prevalence of iridovirus
30 exposure in free-ranging gopher tortoises (*Gopherus polyphemus*) in the southeast United States,
31 an indirect enzyme-linked immunosorbent assay (ELISA) was developed and used to evaluate
32 plasma samples from wild gopher tortoises (*Gopherus polyphemus*) from: Alabama (n=9);
33 Florida (n=658); Georgia (n=225); Louisiana (n=12); Mississippi (n=28); and unknown locations
34 (68) collected between 2001 and 2006. Eight (1.2%) seropositive tortoises were identified from
35 Florida and seven (3.1%) from Georgia for an overall prevalence of 1.5%. Additionally, a
36 population of Eastern box turtles was sampled from a private nature sanctuary in Pennsylvania
37 that experienced an outbreak of iridovirus the previous year, which killed 16 turtles. Only one
38 turtle out of 55 survivors tested positive (1.8%). Results suggest a low exposure rate in
39 chelonians to this pathogen; however, it is suspected that this is an underestimate of the true
40 prevalence. Since experimental transmission studies and past outbreaks have shown a high rate
41 of mortality in infected turtles, turtles may die before they develop an antibody response.
42 Further, the duration of the antibody response is unknown and may also cause an underestimate
43 of the true prevalence.

44

45

46 *Key words:* Reptile; Turtles; Iridovirus; Ranavirus; Enzyme-linked immunosorbent assay

47 1. Introduction

48 Iridoviruses of the genus *Ranavirus* are well known for causing mass mortality events of
49 fish and amphibians with increasing reports of infection in reptiles. Prior to 2003, only two
50 occurrences of chelonian infection with a *Ranavirus* were known in the United States; a free-
51 ranging gopher tortoise (*Gopherus polyphemus*) in Florida (Westhouse et al., 1996) and a
52 collection of captive box turtles (*Terrapene carolina carolina*) in North Carolina (DeVoe et al,
53 2004). Since then, several additional cases and outbreaks have been identified and described in
54 both free-ranging native and captive exotic chelonian species including Eastern and Florida box
55 turtles (*Terrapene carolina carolina* and *T. carolina bauri*), gopher tortoises, and Burmese star
56 tortoises (*Geochelone platynota*) (Allender et al., 2006; Johnson et al., 2008). Experimental
57 transmission studies have confirmed that ranaviruses are in fact primary pathogens of chelonians
58 (Johnson et al., 2007). The method of transmission has yet to be elucidated, but conspecific
59 amphibians have been shown to be infected with identical or very closely related viruses,
60 suggesting that amphibians may serve as a source of infection for chelonians (Johnson et al,
61 2008). The prevalence and incidence of infections in free-ranging chelonians remains unknown.

62 Indirect enzyme linked immunosorbent assays (ELISA) have been used to detect
63 exposure of various species of reptiles to specific pathogens (Schumacher et al., 1993; Origgi et
64 al., 2001; Brown et al., 2001; Jacobson et al., 2005) and have been used to detect exposure of
65 amphibians to iridovirus infections (Whittington et al., 1997; Gantress et al., 2003; Maniero et
66 al., 2006). To determine iridovirus exposure in free-ranging gopher tortoises in the United States,
67 an indirect ELISA was developed using a previously described mouse anti-desert tortoise IgY
68 monoclonal antibody as the secondary antibody (Schumacher et al., 1993). We describe the assay
69 and the results of a larger serological survey of free-ranging gopher tortoises from various sites
70 in Alabama, Florida, Georgia, Louisiana and Mississippi. Additionally, 55 surviving Eastern box

71 turtles (*Terrapene carolina carolina*) from a population in Pennsylvania that experienced an
72 outbreak of illness that killed 16 turtles were sampled to determine whether any of the box turtles
73 were exposed that might have survived infection.

74 **2. Materials and Methods**

75 *2.1 Positive and Negative Reference Plasma and Virus Isolate*

76 In July of 2003, three of five captive Burmese star tortoises (BST) became ill with
77 clinical signs consisting of nasal discharge, conjunctivitis, frothing from the mouth, cervical
78 subcutaneous edema, and oral plaques. One of the three tortoises died and histologic lesions were
79 observed that were consistent with those seen in iridovirus infections in fish, amphibians and
80 other reptiles, including the presence of intracytoplasmic inclusion bodies (Reddacliff et al,
81 1996; Westhouse et al, 1996; Bollinger et al, 1999; Docherty et al, 2003;). Molecular
82 investigations demonstrated the presence of *Ranavirus* in various tissues (Johnson et al, 2008).
83 This *Ranavirus* here termed BSTRV, was used as the antigen in the development of the ELISA.
84 Surviving tortoises were treated with acyclovir (Glaxo Wellcome, Brentford, Middlesex, United
85 Kingdom) and supportive care and all four tortoises survived. Plasma was collected at the time of
86 the onset of clinical signs of disease during July and then again two months later in September
87 2003. Plasma from one of these tortoises collected in September was used as the positive control
88 in development of the ELISA. Plasma from a Burmese star tortoise from a zoological collection
89 with no known history of disease was collected to serve as negative reference plasma for the
90 ELISA.

91 *2.2 Antigen Preparation*

92 *Terrapene* heart-1 (TH-1) cells were grown to confluency in 225cm² tissue flasks (Costar,
93 Corning, NY). Cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco,
94 Carlsbad, CA) supplemented with 5% fetal bovine serum (Gibco), gentamicin (60mg/liter;

95 Sigma, St. Louis, MO), penicillin G (120,000 U/liter), streptomycin (120,000 U/liter) and
96 amphotericin B (300µg/liter; Sigma). Cells were inoculated with a fourth passage of BSTRV and
97 incubated at 28°C in the presence of 5% CO₂. When cytopathic effects (CPE) consisting of cell
98 rounding and detachment from the flask were observed in over 80% of cells, the cells and media
99 were transferred to 15ml tubes and centrifuged at 4,500xg for 30 minutes. Supernatant was then
100 discarded and the cell pellets were resuspended in residual media, and frozen and thawed three
101 times to release virus from the cells. Tubes were vortexed before and after each freeze cycle and
102 following the final thaw. Subsequently, they were centrifuged again at 4,500xg for 30 minutes.
103 Supernatant containing concentrated amounts of virus was then transferred to a 4ml sterile
104 cryotube. A Bradford protein assay was performed to determine the final protein concentration of
105 the antigen (Bradford, 1976; BioRad, Hercules, CA). A previously described polymerase chain
106 reaction (PCR) test was used to confirm the presence of the *Ranavirus* major capsid protein
107 (MCP) gene (Marschang et al, 1999). Uninfected flasks were concurrently processed in the same
108 manner to serve as control antigen to detect any background cross reactivity of plasma to cellular
109 proteins.

110 2.3 ELISA Procedure

111 A checkerboard optimization strategy was used to determine the optimum concentrations
112 of both antigen and plasma to be used in the ELISA. Antigen concentrations were evaluated at
113 dilutions of 1:100, 1:250, 1:500, and 1:1000. Plasma concentrations evaluated were two-fold
114 serial dilutions from 1:50 to 1:1600. The following procedure was found to be optimal utilizing
115 the crude cell lysate antigen. Each well of a high protein binding 96 well microplate (Maxisorp
116 F96; Nunc, Kamstrup, Denmark) was coated with 50µl of infected or uninfected cell lysate
117 diluted to 1:100 in 0.01M sodium phosphate buffer (pH 7.2) containing 0.15 M NaCl and 0.02%

118 sodium azide (PBS/Az). Plates were incubated overnight at 4°C. Antigen was then aspirated off
119 and wells were washed four times with ELISA wash buffer (PBS/Az with 0.05% Tween 20).
120 This washing process was performed between each of the following steps. Wells were then
121 blocked against non-specific binding with 300µl of Superblock blocking buffer by Pierce
122 (Rockford, IL) for one hour at room temperature (RT). Each remaining step was incubated for
123 1hr at RT. Plasma samples diluted 1:100 in blocking buffer were added at 50µl volumes to wells
124 in triplicate. One well was coated with uninfected cell lysate, while the other two wells were
125 coated with infected cell lysate. Next, a biotin-conjugated monoclonal antibody produced against
126 the desert tortoise IgY light chain (Schumacher et al., 1993) was diluted to a final concentration
127 of 0.5µg/ml in PBS/Az and added to each well in 50µl volumes. Alkaline phosphatase-
128 conjugated streptavidin (Zymed Laboratories, Inc., San Francisco, California) was applied to
129 each well at 50µl of a 1:5000 dilution in PBS/Az. Next, the ELISA was developed with 100µl
130 per well of a 1.0mg/ml *P*-nitrophenyl phosphate prepared in 0.01M sodium bicarbonate buffer
131 containing 2mM MgCl₂ and plates were stored in the dark. The absorbance of each well was read
132 at A₄₀₅ using a StatFax 3200 microplate reader (Awareness Technology, Palm City, Florida) at
133 30 minutes.

134 Each plasma sample was read in triplicate. Plasma was placed on one well originally
135 coated with uninfected cell lysate and on two wells coated with infected cell lysate. The average
136 absorbance reading of the two wells coated with infected cell lysate was calculated and the
137 positive/negative (P/N) ratio value of each sample was determined by dividing the mean
138 absorbance of the duplicate average by the absorbance reading of the well from the uninfected
139 cell lysate. This subtracts out any background noise, or non-specific binding that may have
140 occurred as a result of using the *Terrapene* heart cell line. The cut-off value for a positive test

141 result was determined by adding three times the standard deviation to the mean P/N ratio
142 obtained (Crowther, 2001).

143 *2.4 Reproducibility*

144 Intra-assay and inter-assay reproducibility were determined by performing two precision
145 runs. The positive and negative reference plasma samples, used to optimize the test, were used in
146 each assay. Intra-assay reproducibility was determined by running the positive and negative
147 sample multiple times on the same plate. Each sample was run multiple times on one well of
148 uninfected cell lysate and two wells of infected cell lysate. This resulted in 64 readings for each
149 sample on the infected cell lysate and 32 on the uninfected cell lysate. Inter-assay reproducibility
150 was determined by using the values of the reference plasma results used as controls in assaying
151 the wild gopher tortoise samples from multiple dates and multiple plates. The mean A_{405} , the
152 standard deviation (SD) and the coefficient of variation (CV) for the intra- and inter-assay
153 reproducibility were calculated using the optimized ELISA conditions.

154 *2.5 Protein Expression and Immunoblotting*

155 The positive control plasma was tested for its ability to detect viral proteins in a western
156 blot using infected and uninfected TH-1 cell lysates as antigen. Four four-fold dilutions of
157 infected and uninfected cellular lysate proteins were separated by sodium dodecyl sulfate-
158 polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions along with broad
159 range molecular weight markers. The separated proteins were transferred onto 0.2 μ m
160 nitrocellulose membranes (Biorad, Hercules, CA) by standard methods (Harlow and Lane,
161 1988). Membranes were then rinsed in water for five minutes in preparation for coomassie blue
162 staining or in tris buffered saline containing 0.5% Tween20 (TTBS, pH 7.5) for twenty minutes
163 prior to immunoblotting. Membranes were then stained in coomassie blue stain for 90 minutes,
164 followed by water for 30 minutes to destain to view differences in protein profiles between

165 infected and uninfected cell lysates. After rinsing in TTBS, membranes for immunoblotting were
166 blocked with Superblock blocking buffer in phosphate buffered saline (Pierce) for one hour.
167 Blocking buffer was then removed and plasma samples diluted 1:2000 in blocking buffer were
168 added to the membranes. After one hour, the membranes were washed with TTBS for 30 minutes
169 and the monoclonal antibody, diluted 1:10,000 in blocking buffer, was added and incubated for
170 one hour. Again the membranes were washed and AP streptavidin, diluted 1:5,000 in phosphate
171 buffered saline was added and allowed to incubate for one hour. Membranes were washed as
172 previously described, and the membranes were developed in substrate buffer (0.1M Tris-HCl,
173 1mM MgCl₂) containing nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-
174 indolylphosphate *p*-toluidine salt (BCIP) (Biorad). The reaction was stopped by removing the
175 NBT-BCIP solution and adding deionized water. Membranes were allowed to air dry.

176 *2.6 Free-ranging Gopher Tortoise Samples*

177 Plasma samples from 1000 wild gopher tortoises (*Gopherus polyphemus*) from Alabama
178 (n=9), Florida (n=658), Georgia (n=225), Louisiana (n=12), Mississippi (n=28) and unknown
179 locations (68) were obtained from recently submitted samples to the Mycoplasma Research
180 Laboratory at the University of Florida between 2001 and 2006. County, state and date of sample
181 collection was recorded for each plasma sample when it was available.

182 *2.7 Box Turtle Samples*

183 During the summer and fall of 2003, a population of 71 repatriated Eastern box turtles
184 (*Terrapene carolina carolina*) in a private nature sanctuary in Pennsylvania, regularly tracked by
185 radiotelemetry for over four years, experienced an unprecedented occurrence of sudden deaths in
186 16 turtles. The outbreak was investigated and iridovirus infections were confirmed in three of the
187 turtles as previously described (Johnson et al., 2008). It is suspected to be the cause of death in
188 the other 13 turtles, however further diagnostic work-up was not performed in these turtles. In

189 2004, the surviving 55 turtles were sampled. Plasma samples were stored on ice and transported
190 to the University of Florida for testing.

191

192 **3. Results**

193 *3.1 ELISA Parameters*

194 Results of the checkerboard optimization strategy determined that a 1:100 dilution of
195 both the antigen and plasma samples gave the largest difference of P/N ratios between the
196 positive and negative reference samples (Fig. 1). The frequency distribution of the P/N ratio is
197 shown in 0.1 increments from 0.5 to 4.1 (Fig. 2). The mean P/N ratio of samples (1.078), plus
198 three times the standard deviation (0.379) was used to set the positive cut-off value at 2.2. P/N
199 ratios from each sample were plotted on a graph with the sample numbers on the x-axis in
200 increasing order and the P/N ratio values on the y-axis (Fig. 3).

201 *3.2 Reproducibility*

202 The mean A_{405} , SD and CV values for the intra- and inter-assay precision runs are shown
203 in Table 1. Ideally, CV values should be less than 15% (Crowther, 2001). Three of the CV values
204 in the inter-assay precision runs were >15% indicating that some variability still existed in this
205 assay when performed at different time periods. Intra-assay coefficients of variance were less
206 than 15%.

207 *3.3 Protein Expression and Immunoblotting*

208 Coomassie blue staining of proteins from infected cell lysates and uninfected cell lysates
209 showed a different pattern of protein expression (Fig. 4). Immunoblotting of infected and
210 uninfected cell lysate showed a marked increase in binding to proteins in the infected cell lysate
211 and very weak binding to proteins in the uninfected cell lysate (Fig. 5). Strong signals were seen

212 on virus-infected cells at approximately 125 kDa, and 78 kDa with weaker signals seen at 70, 65,
213 and 28 kDa. Very faint signals were also seen at 125 and 78 kDa in the uninfected cells.

214 *3.4 Free-ranging Gopher Tortoise Samples*

215 Of 1000 gopher tortoise plasma samples assayed, eight (1.2%) tortoises from Florida and
216 seven (3.1%) from Georgia were seropositive, for a total of 1.5% of tortoises seropositive with a
217 P/N ratio >2.2 (Table 2). All tortoises from Alabama, Louisiana and Mississippi were negative,
218 as were the ones from unknown locations. Seropositive tortoises in Florida were located in five
219 counties including Lake, St. Lucie, Broward, Palm Beach and Martin (Table 3, Fig. 6). Four of
220 these are clustered together in the south including Palm Beach, Broward, Martin, and St. Lucie.
221 The remaining seven seropositive tortoises were located in Baker, Georgia, a county in the
222 southwest corner of the state (Fig. 6).

223 Date of sample collection was recorded in 680 samples. Two thirds of the positive samples
224 were collected in 2005 (Table 4), of which seven were from tortoises in Baker County, Georgia.
225 One positive sample each was collected in 2003 and 2004, two positive samples in 2006 and no
226 samples tested positive from those collected in 2001 and 2002.

227 *3.5 Box turtle Samples*

228 Of 55 samples, only one box turtle tested positive with a P/N ratio of 7.2 for a prevalence
229 of 1.8%. All other samples had a P/N ratio less than 1.7. No clinical signs of illness were
230 observed in the turtle that tested positive, and the turtle, which was being regularly tracked by
231 radiotelemetry had no known history of illness.

232

233 **4. Discussion**

234 This study documents the development of an assay to detect anti-iridovirus antibodies and
235 the first report of anti-iridovirus antibodies in free-ranging and captive chelonians. The ELISA

236 detected anti-iridovirus antibodies two months after two Burmese star tortoises recovered from
237 an upper respiratory illness with oral plaques, which had been previously housed with a tortoise
238 that died with confirmed iridovirus infection (Johnson et al, 2008). Plasma from a Burmese star
239 tortoise from another facility with no known history of illness was selected as a seronegative
240 control and no antibodies to iridovirus were detected in this tortoise. Additionally, using a
241 western blot, we were able to show that plasma from the positive control turtle sample bound to
242 proteins in the infected cells in the immunoblot, but not in the uninfected cells indicating that the
243 plasma was reacting with viral proteins and not cellular proteins from the lysate that was used to
244 coat the plates. For these reasons, we consider this a valid test for use in detecting anti-iridovirus
245 antibodies in chelonians, however further validation of this assay against another diagnostic
246 assay is warranted to establish the sensitivity and specificity of the assay. Additionally, inter-
247 assay precision runs indicated that some variability still exists when the assay is run at different
248 time periods. The values for samples run on infected cell lysate were more variable than those
249 run on uninfected cell lysate. This suggests that the technique for preparing antigen may vary,
250 either due to differences because of the operator, or in the assay and/or reagents.

251 Humoral immune responses are characterized by an early response as indicated by an acute
252 increase in plasma IgM levels, followed by an increase in IgG levels in mammals, or IgY levels
253 in reptiles. Early studies using the anti-desert tortoise IgY light chain monoclonal antibody found
254 that the antibody would detect the light chains of both IgM and IgY (Schumacher et al, 1993).
255 This suggests that this ELISA can detect both recent infections with Iridovirus as well as
256 infections that may have occurred awhile before the plasma was collected. In the same study by
257 Schumacher et al., seroconversion following inoculation with *Mycoplasma agassizii* occurred
258 between 1 and 3 months (Schumacher et al, 1993), suggesting that in animals with positive

259 ELISA results, exposure to iridovirus occurred at least one month prior to the date the sample
260 was collected.

261 Prevalence among free-ranging gopher tortoises utilizing this assay was found to be low;
262 only 1.5% of 1000 samples were positive. This could be the true prevalence rate, although we
263 suspect that this is an underestimation. Prevalence is a function of the incidence of disease
264 multiplied by the average duration of the illness. While incidence would be difficult to determine
265 in a natural setting, duration of illness can be extrapolated from experimental studies. If
266 chelonians die quickly as a result of infection, they will not have time to develop an immune
267 response to the pathogen, and will not survive to be surveyed. As previously mentioned,
268 experimental transmission studies have shown a high rate of mortality (75%) in turtles
269 intramuscularly inoculated with 1 ml of 10^5 TCID₅₀/ml of an iridovirus-infected cell lysate
270 (Johnson et al., 2007). Turtles that succumbed to the virus died within 30 days of exposure. If
271 naturally exposed cases experience similar mortality rates and duration from exposure to death, a
272 cross-sectional study evaluating the prevalence of exposure will miss many tortoises that were
273 exposed, because most tortoises will die. This was demonstrated in a natural setting in
274 Pennsylvania. Seventy-one radiotelemetered eastern box turtles (*Terrapene carolina carolina*)
275 were being followed by researchers since 1999 in a private nature sanctuary. Turtles had been
276 translocated from another preserve in 1999 and experienced no disease outbreaks until 2003. In
277 the summer and fall of 2003, 16 of these turtles died suddenly, with what was later identified to
278 be iridovirus infections (Johnson et al., 2008). The following spring, plasma from the surviving
279 55 turtles was evaluated by ELISA for the presence of anti-iridovirus antibodies. Only one turtle
280 was positive on ELISA. If we calculated this value as the true prevalence, we would estimate that
281 about 1.8% (1/55) of turtles were exposed, when we know that 24% (17/71) of the population

282 was actually exposed, but 22% (16/71) died and so were not available to be included in the
283 estimate. This severely underestimates the prevalence of disease. However, we cannot
284 extrapolate these differences to other populations, as this was a repatriated population, and thus,
285 subject to stressors that might not be found in naturally occurring populations, making them
286 more susceptible to disease. Additionally, we are unsure how long anti-iridovirus antibodies
287 remain at detectable levels in chelonians, or if all animals infected with the virus will mount an
288 antibody response. If antibody levels decline prior to testing, or they never develop, then animal
289 test results may be falsely negative, also creating an underestimate of prevalence.

290 Results of this serosurvey showed that counties in the southeastern region of Florida were
291 more likely to have seropositive tortoises. Interestingly, four of these counties had adjoining
292 borders (St. Lucie, Martin, Palm Beach and Broward), suggesting that either tortoises in this area
293 are exposed to iridoviruses at a higher rate, or tortoises in this area are more resistant to disease,
294 due to genetic or environmental factors. Environmental persistence of iridoviruses is poorly
295 understood, however it appears moisture and temperature may play important roles. Studies with
296 insect iridoviruses found that moisture was associated with increased persistence, while
297 desiccation inactivated viruses (Williams et al., 2005). Temperature has also been shown to play
298 an important role in the infectivity of salamanders to iridoviruses, where salamanders exposed at
299 lower temperatures are more susceptible to the virus than those infected at higher temperatures
300 (Rojas et al. 2005). It is possible that tortoises infected with the virus in a warmer climate may be
301 more resistant to infection, allowing them to develop an immune response and survive. However,
302 this would not account for the higher prevalence rate of exposure of tortoises tested in Baker
303 County, Georgia. Direct transmission may play a role in infection rates. In amphibians, activities
304 involving close contact have been shown to be effective means of transmitting *Ranavirus*

305 (Brunner et al., 2007). Box turtles have been shown to have overlapping home ranges, and
306 mating occurs among turtles with overlapping ranges (Stickel, 1950; Stickel 1978; Stickel 1989).
307 Interactions between males are similar to courtship behaviors between males and females
308 (Stickel, 1989) and box turtles are often found near other box turtles (Dodd, 2001). Gopher
309 tortoises of opposite sex often have overlapping home ranges and can commonly occupy the
310 same burrow (Diemer, 1992). Therefore, it is possible that differences in population densities or
311 overlapping home ranges may play a role in increased levels of exposure.

312 Earlier studies have shown that amphibians carry identical or very closely related
313 iridoviruses to those found in the chelonians, suggesting they might be a source of infections
314 (Johnson et al., 2008). Green et al. (2002) found that increased precipitation and population
315 densities were directly associated with increased die-offs of amphibians due to iridoviruses.
316 Other conditions that may cause stress and compromise the immune system have also been
317 shown to increase susceptibility to iridoviruses. Forson and Storfer (2006) found that atrazine
318 and sodium nitrate resulted in decreased peripheral leukocyte levels and increased susceptibility
319 of salamanders to a *Ranavirus*. Gray et al (2007) found that in some species of amphibians, stress
320 resulting from sharing wetlands with cattle resulted in increased susceptibility of tadpoles to
321 iridovirus infection. Similar conditions that cause stress to chelonians might also result in
322 increased levels of susceptibility in chelonians. Additionally, it has been shown that sublethally
323 infected amphibians can cause sporadic, recurrent disease outbreaks in amphibian populations
324 (Brunner et al., 2004). Experimentally and naturally infected tiger salamander larvae and
325 metamorphs were able to maintain sublethal, transmissible infections for over five months.
326 Apparently healthy infected dispersing metamorphs were returning to water bodies to breed and
327 it was speculated that these individuals were likely serving as a reservoir host for infecting newly

328 hatched larvae, creating recurrent outbreaks of disease. It is unknown whether chelonians are
329 capable of sustaining a subclinical persistent infection. This assay has conservation value since it
330 can be used to identify asymptomatic carriers in wild and captive populations.

331 **5. Conclusion**

332 This study reports the development and use of an indirect ELISA for detection of anti-
333 iridovirus antibodies in chelonians. Overall seroprevalence of exposure of free-ranging gopher
334 tortoises to iridoviruses appear to be low, however, this is expected to be an underestimate of the
335 true prevalence. Further studies are needed to characterize the impact of this viral disease on
336 gopher tortoises and other chelonian species.

337

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346

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427 Virus Res. 65, 173-248.

428 Table 1. Reproducibility of the ELISA. SD = standard deviation of the mean A_{405} . CV =
 429 coefficient of variance expressed as a percent. ICL = values from samples run on wells
 430 coated with infected cell lysate. UCL = values from samples run on wells coated with
 431 uninfected cell lysate.

432

		Positive Sample				Negative Sample			
		n	Mean A_{405}	SD	CV	n	Mean A_{405}	SD	CV
Intra-assay	ICL	64	0.363	0.022	6.06	64	0.107	0.011	10.28
	UCL	32	0.094	0.009	9.57	32	0.112	0.009	8.04
Inter-assay	ICL	26	0.366	0.079	21.58	26	0.130	0.023	17.69
	UCL	13	0.095	0.011	11.58	13	0.111	0.017	15.31

433

434

434 Table 2. ELISA results of plasma samples from free-ranging gopher tortoises (*Gopherus*
435 *polyphemus*) by state.

436

State	Total No. Tested	Total No. Positive	Percent
Alabama	9	0	0
Florida	658	8	1.2
Georgia	225	7	3.1
Louisiana	12	0	0
Mississippi	28	0	0

437

438

439

440

440 Table 3. Anti-iridovirus ELISA results of 1000 free-ranging gopher tortoise (*Gopherus*
 441 *polyphemus*) plasma samples by county.

State	County	Number tested	Number Positive	Percent
Alabama	Baldwin	2	0	0
	Mobile	7	0	0
Florida	Alachua	12	0	0
	Brevard	27	0	0
	Broward	10	1	10
	Citrus	51	0	0
	Clay	1	0	0
	Collier	2	0	0
	Columbia	1	0	0
	Hernando	24	0	0
	Hillsborough	16	0	0
	Indian River	2	0	0
	Lake	99	3	3
	Lee	18	0	0
	Leon	17	0	0
	Madison	1	0	0
	Manatee	6	0	0
	Marion	22	0	0
	Martin	25	1	4
	Miami-Dade	8	0	0
	Nassau	47	0	0
	Orange	40	0	0
	Osceola	11	0	0
	Palm Beach	58	2	3
	Pasco	26	0	0
	Pinellas	2	0	0
	Polk	6	0	0
	Sarasota	1	0	0
	Seminole	54	0	0
	St. Johns	7	0	0
	St. Lucie	35	1	3
	Taylor	1	0	0
Volusia	26	0	0	
Walton	2	0	0	
Georgia	Baker	113	7	6
	Liberty	74	0	0
	Tattnall	38	0	0

Louisiana	Washington Parish	12	0	0
Mississippi	Greene	7	0	0
	Harrison	16	0	0
	Perry	5	0	0
	Unknown	68	0	0
TOTAL		1000	15	1.5

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443 Table 4. Total number of samples collected each year and those that tested positive for
 444 anti-iridovirus antibodies with the county and state.

Year	Number Tested	Number Positive	County, State
2001	4	0	
2002	84	0	
2003	189	1	Palm Beach, FL
2004	124	1	Lake, FL
		7	Baker, GA
2005	178	1	Lake, FL
		1	Martin, FL
		1	St. Lucie, FL
2006	101	1	Broward, FL
		1	Palm Beach, FL
Unknown	320	1	Lake, FL

445

446

446 **Figure Captions**

447 Fig. 1. Optimization of the ELISA with antigen coated at 1:100 dilution, comparing
448 the positive to negative (P/N) ratio of two fold serial plasma dilutions of the
449 positive control turtle (Burmese star tortoise with clinical signs of illness)
450 versus a negative control (Burmese star tortoise with no history of illness).
451 Plasma diluted at 1:100 showed the greatest difference between the positive
452 and negative control.

453 Fig. 2. Frequency distribution of P/N ratios from an indirect ELISA performed on 1000
454 free ranging gopher tortoise (*Gopherus polyphemus*) plasma samples.

455 Fig. 3. Individual P/N ratio values for 1000 free-ranging gopher tortoises (*Gopherus*
456 *polyphemus*) in increasing value.

457 Fig. 4. Coomassie blue staining of a SDS-PAGE gel separating proteins of iridovirus-
458 infected and uninfected *Terrapene* heart cell lysates. Lane 1 is a broad range,
459 pre-stained molecular weight marker with weight in kDa marked next to the
460 lane. Lanes 2-5 are four fold serial dilutions of iridovirus infected cell lysate.
461 Lanes 6-8 are four fold serial dilutions of uninfected cell lysate.

462 Fig. 5. Western immunoblot. Lanes coated with four fold serial dilutions of iridovirus
463 infected cell lysate in lanes 2-5 and uninfected cell lysate in wells 6-9. Plasma
464 from the positive control was used as the primary antibody for detection of
465 iridovirus specific antibody binding. Strong signal was seen on virus-infected
466 cells at approximately 125 kDa, and 78 kDa with weaker signals seen at 70,

467 65, and 28 kDa. Very faint signals were also seen at 125 and 78 kDa in the
468 uninfected cells.

469 Fig. 6. Map showing counties in Alabama, Florida, Georgia, Louisiana and Mississippi.

470 Counties highlighted in light gray represent the counties where tortoise samples
471 originated. Counties in dark gray had tortoises tested that were seropositive
472 for exposure to an iridovirus.

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Figure 1

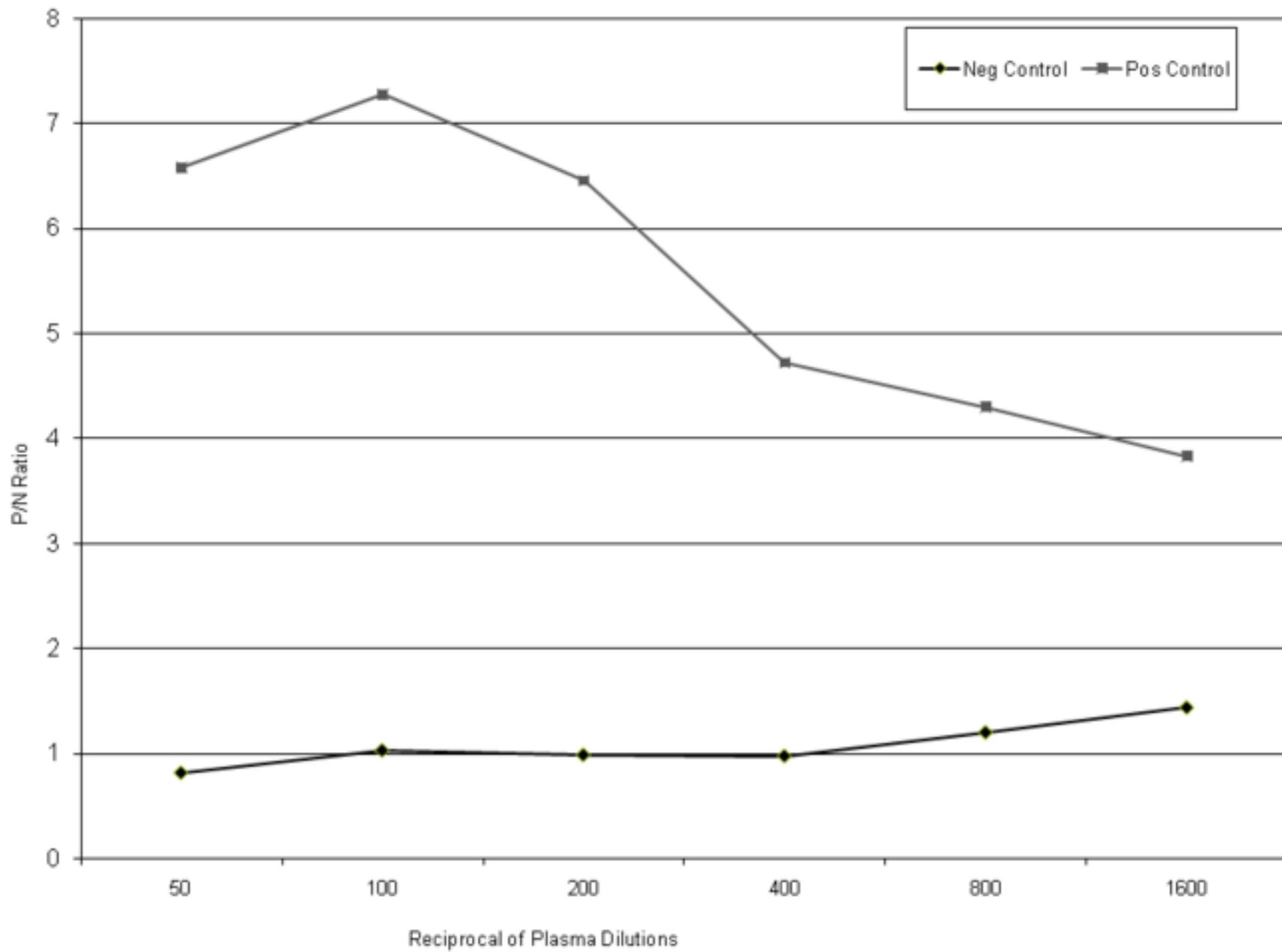


Figure 2

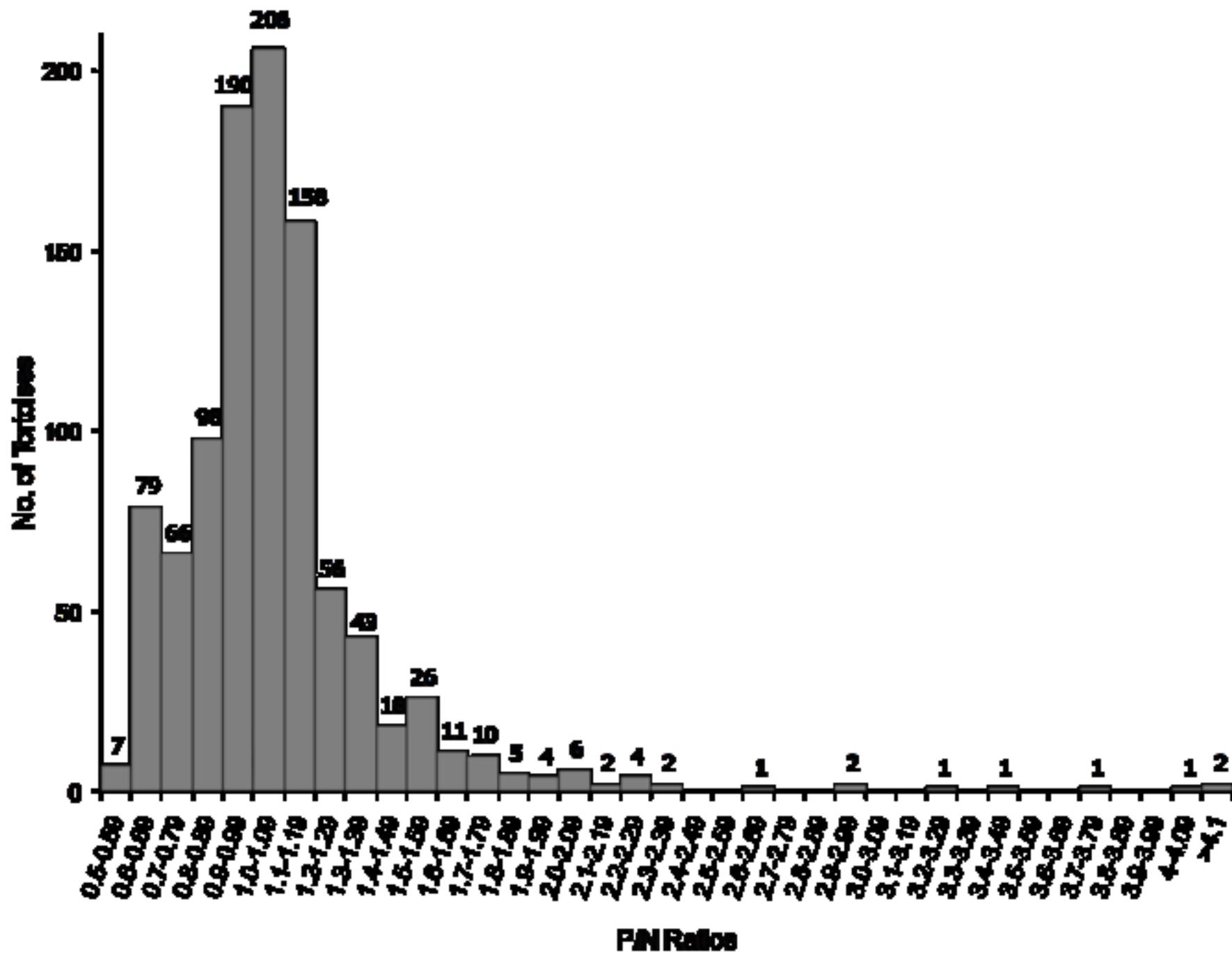


Figure 3

trip

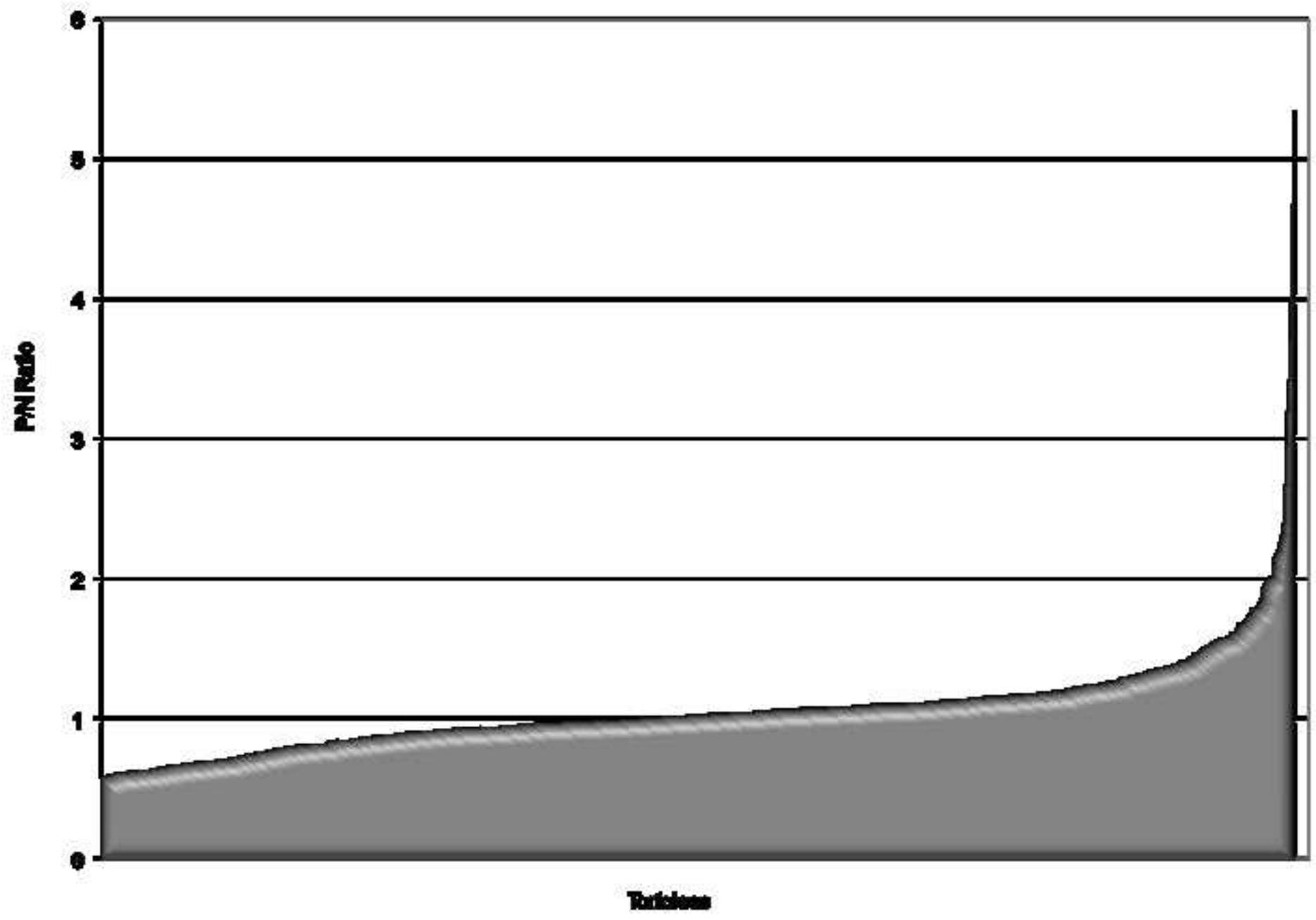


Figure 4

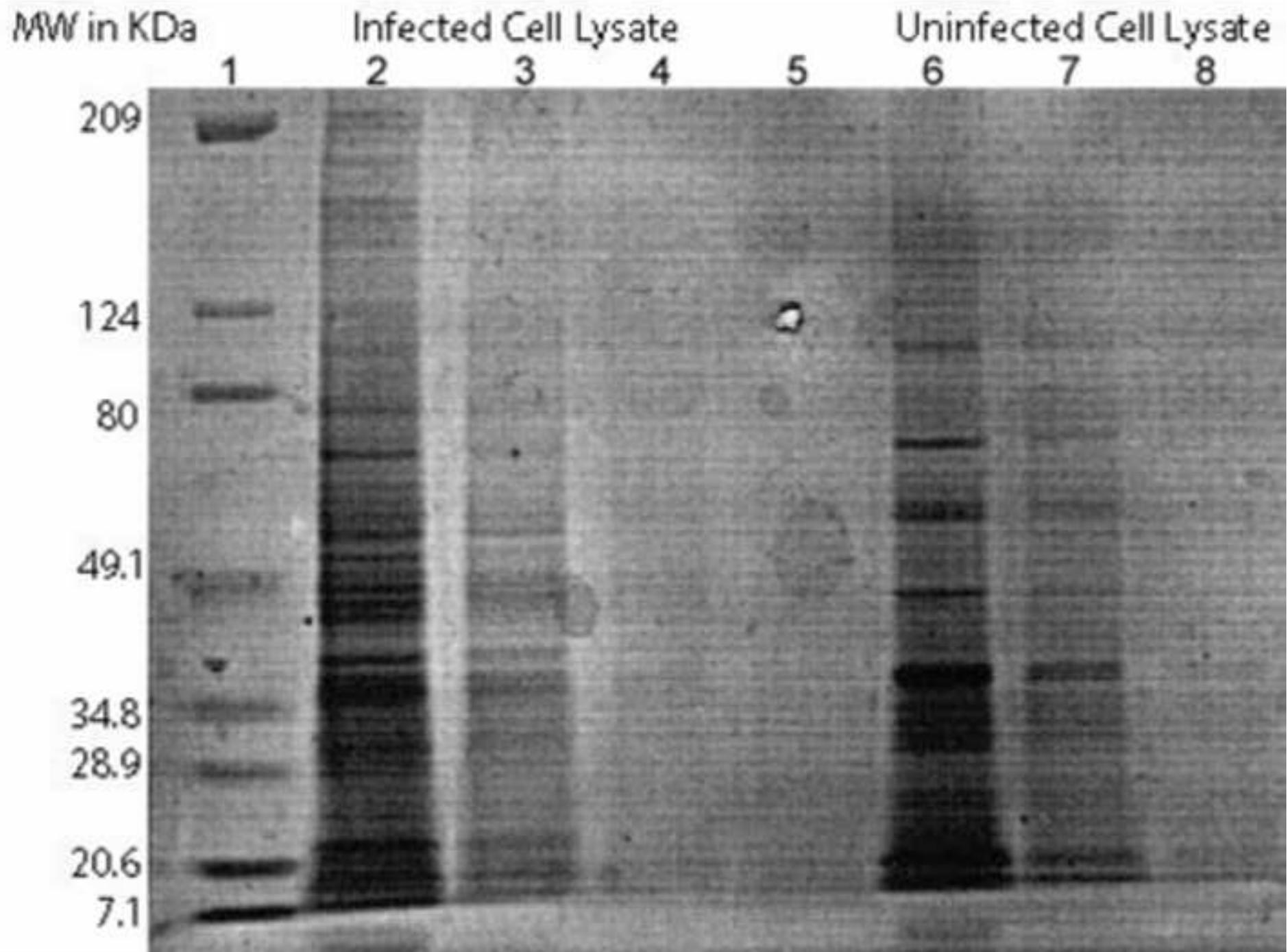
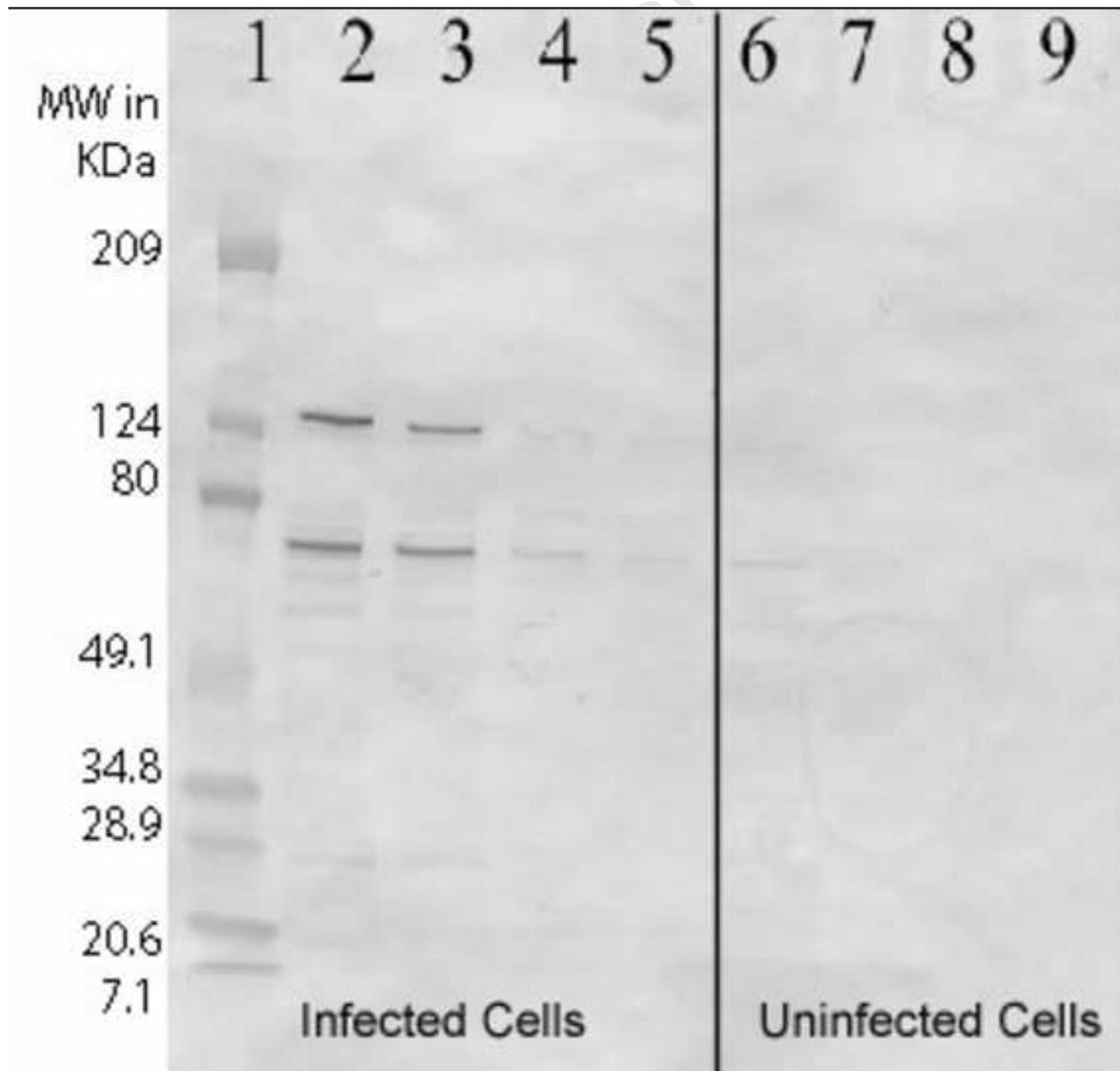


Figure 5



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