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## Detection of specific antibody responses to vaccination in variable flying foxes (*Pteropus hypomelanus*)

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### Abstract

Megachiropteran bats are biologically important both as endangered species and reservoirs for emerging human pathogens. Reliable detection of antibodies to specific pathogens in bats is thus epidemiologically critical. Eight variable flying foxes (*Pteropus hypomelanus*) were immunized with 2,4-dinitrophenylated bovine serum albumin (DNP-BSA). Each bat received monthly inoculations for 2 months. Affinity-purified IgG was used for production of polyclonal and monoclonal anti-variable flying fox IgG antibodies. ELISA and western blot analysis were used to monitor immune responses and for assessment of polyclonal and monoclonal antibody species cross-reactivity. Protein G, polyclonal antibodies, and monoclonal antibodies detected specific anti-DNP antibody responses in immunized variable flying foxes, with protein G being the most sensitive, followed by monoclonal antibodies and then polyclonal antibodies. While the polyclonal antibody was found to cross-react well against IgG of all bat species tested, some non-specific background was observed. The monoclonal antibody was found to cross-react well against IgG of six other species in the genus *Pteropus* and to cross-react less strongly against IgG from *Eidolon helvum* or *Phyllostomus hastatus*.

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32  
33 Protein G distinguished best between vaccinated and unvaccinated bats, and these results validate the  
34 use of protein G for detection of bat IgG. Monoclonal antibodies developed in this study recognized  
35 immunoglobulins from other members of the genus *Pteropus* well, and may be useful in applications  
36 where specific detection of *Pteropus* IgG is needed.

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40  
41 *Keywords:* Bats; Flying fox; *Pteropus hypomelanus*; Antibody; Protein G; ELISA

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## 42 Résumé

43 Les chauves-souris du groupe mégachiroptère sont importantes au point de vue biologique, parce  
44 qu'elles représentent un réservoir pour de nouvelles maladies humaines. La possibilité de détecter  
45 leurs anticorps contre des pathogènes spécifiques est donc critique au point de vue épidémiologique.  
46 Huit roussettes des îles (*Pteropus hypomelanus*) furent injectées avec de l'albumine de sérum bovin  
47 (2,4-dinitrophenylated bovine serum albumin, DNP-BSA) deux fois, à quatre semaines d'intervalle.  
48 Les anticorps IgG à affinité purifiée furent utilisés pour produire les anticorps mono- et polyclonaux  
49 IgG de chauves-souris. La réponse immunitaire des animaux et la réaction croisée entre les anticorps  
50 de différentes espèces furent mesurés avec l'ELISA et l'analyse de Western blot.

51 Au jour 56 après l'injection, une magnification de la densité optique (optical density, OD<sub>405</sub>) entre  
52 4 et 17 fois a été mesurée lorsque la protéine G fut utilisée comme agent. Quand des anticorps  
53 polyclonaux de lapins souris furent utilisés, la magnification fut de 1.4 à 2, alors que lorsque des  
54 anticorps monoclonaux de souris furent utilisés, la magnification fut de 1.7 à 7.

55 La protéine G, les anticorps mono et polyclonaux ont réussi à détecter une réponse immunitaire  
56 contre l'agent DNP chez les roussettes. Malgré que les anticorps polyclonaux aient réussi à faire une  
57 réaction croisée avec les IgG des chauves-souris, il y avait aussi beaucoup d'interférences.

58 Les anticorps monoclonaux ont très bien réagi avec 6 autres espèces du genre *Pteropus*, mais  
59 moins bien avec les espèces *Eidolon helvum* ou *Phyllostomus hastatus*. La protéine G fut utile pour  
60 distinguer les animaux vaccinés des naïfs, et ces résultats valident l'utilisation de la protéine G pour la  
61 détection des anticorps IgG chez les chauves-souris.

62 Les anticorps monoclonaux développés dans ce projet ont aussi pu détecter les immunoglobulines  
63 de d'autres membres de la famille *Pteropus*, et leur usage pourrait être utile dans certains cas où l'on a  
64 besoin de détecter les anticorps IgG.

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66 *Mots clés :* Chauves-souris ; Roussette ; *Pteropus hypomelanus* ; Anticorps ; Protéine G ; ELISA

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## 67 1. Introduction

68 Bats are the second largest mammalian order, Chiroptera, with approximately 1100  
69 species [1]. The variable flying fox, *Pteropus hypomelanus*, is a megachiropteran bat that  
70 inhabits Sulawesi and small islands from the Bay of Bengal and the Malay Peninsula to  
71 New Guinea and the Solomon Islands. The range of this species is fragmented, and rapid  
72 habitat destruction is occurring throughout the range. Since 1989, it has been listed as  
73 CITES appendix II. There are seven subspecies of this bat, the most endangered being *P.*  
74 *hypomelanus maris*, found on the Maldives [2]. The greatest threat to the variable flying fox  
75 is human predation and habitat destruction. This bat is heavily hunted and is exported for  
76 food in the Philippines. Residents of Guam have imported large numbers of *Pteropus* spp.  
77

77  
78 from other Pacific islands as a delicacy. Thirty-four percent of megachiropteran bats are at  
79 risk of extinction [3], and all members of the genus *Pteropus* are listed as at least CITES  
80 appendix II.

81 It is not surprising to find significant pathogen diversity in bats. There are over 900 bat  
82 species, representing approximately 20% of mammalian diversity [4]. While less than 2%  
83 of human pathogens have bats as natural reservoirs, several emerging potentially lethal  
84 viral infections of humans have recently been found in wild flying fox populations,  
85 attracting significant interest [4]. These include Nipah virus, Menangle virus, Hendra virus,  
86 and Australian bat lyssavirus. Evidence suggests that megachiropteran bats are carriers of  
87 Ebola virus [5], and Rhinolophid bats, which are closely related to the megachiropterans,  
88 are carriers of SARS-like coronaviruses [1,6,7]. With the subsequent interest in viruses of  
89 bats, Tioman virus has recently been discovered [8], and it is likely that further bat viruses  
90 will be discovered in the near future. Evidence for Nipah virus [9], Tioman virus, and  
91 Australian bat lyssavirus [10] have been found in wild *P. hypomelanus*. Currently, serum  
92 neutralization assays are the most common tests used to detect the presence of antibodies to  
93 specific pathogens in bats, and are accepted as the reference standard [11]. However, serum  
94 neutralization assays require days to weeks for results, and require virus culture, which is  
95 labor intensive, slow, and involves live virus culture, representing a risk to personnel. IgG  
96 assays have also been performed in bats using chimeric protein G, a streptococcal protein,  
97 and protein A, a staphylococcal protein, that bind IgG from a number of different species  
98 [11–13]. Assays have also been performed using only protein G [7,14]. Protein A and  
99 protein G do not bind well to immunoglobulins from all species [15]. Protein A has been  
100 found not to bind well to immunoglobulins from some bat species [16]. The specificity and  
101 avidity of either protein G or chimeric protein G/protein A for bat immunoglobulins has not  
102 been published.

103 There is not only a need to rapidly assess wild populations for emerging bat and human  
104 pathogens but also a need to establish healthy specific pathogen free captive populations of  
105 fruit bats. While transmission studies may result in the death of the animal being  
106 challenged, immunization studies are rather benign, having minimal affect on the host  
107 other than inducing an immune response. This is an important concern when working with  
108 threatened species. This project used an immunization study to develop and validate the  
109 reagents needed for developing ELISAs for the variable flying fox. These reagents and the  
110 information generated will prove useful for seroepidemiological studies of *Pteropus* spp.

## 111 2. Materials and methods

### 112 2.1. Variable flying foxes and immunization

113 Eight adult (4 male, 4 female) variable flying foxes (*P. hypomelanus*) housed at the  
114 Lubee Bat Conservancy in Gainesville, Florida were used in this study. All were assessed  
115 as healthy before inclusion in the study based on physical examination and blood values  
116 within reference ranges for this species. This protocol was approved by the University of  
117 Florida Institutional Animal Care and Use Committee. Blood samples (2 ml) were  
collected from the brachial vein and placed into lithium heparin tubes on days 0, 28, and 56.

118  
119 Following blood collection on days 0 and 28, six bats (3 male, 3 female) were immunized  
120 by subcutaneous and intramuscular inoculation with 250 µg (500 µl; 1/2 SQ-1/2 IM)  
121 conjugated 2,4-dinitrophenylated bovine serum albumin (DNP-BSA) (Molecular Probes,  
122 Eugene, OR) in a monophosphoryl lipid A plus synthetic trehalose dimycolate  
123 (MPL + TDM) adjuvant system (Sigma Chemical Co., St. Louis, MO). Two bats (1  
124 male, 1 female) were immunized by subcutaneous and intramuscular inoculation with  
125 phosphate-buffered saline (PBS) in MPL + TDM adjuvant as negative controls. Plasma  
126 was obtained by low speed centrifugation of the tubes at  $300 \times g$  for 10 min at room  
127 temperature. All plasma samples were stored at  $-70^{\circ}\text{C}$ .

## 128 2.2. Cross-reactivity of other anti-immunoglobulin reagents with *P. hypomelanus* immunoglobulins

129  
130 To determine the best reagent for purification of *P. hypomelanus* immunoglobulin, an  
131 ELISA looking for binding of anti-IgG reagents to *P. hypomelanus* plasma was performed.  
132 Ninety-six well plates (Nunc Maxisorp, Fisher Scientific, Pittsburgh, PA) were coated with  
133 DNP-BSA overnight at  $4^{\circ}\text{C}$ . Positive control wells were coated with serum appropriate for  
134 the secondary antibody. Wells were washed with PBS containing 0.02% sodium azide and  
135 0.05% Tween-20 using a microplate washer (Biotek Instruments, Winooski, VT). A 1:100  
136 dilution of immunized bat serum in PBS containing 0.02% sodium azide (PBS-AZ) was  
137 added. Negative control wells were incubated with PBS-AZ only. The plates were  
138 incubated at room temperature for 1 h. The plates were washed, and various secondary  
139 alkaline phosphatase-conjugated anti-immunoglobulin reagents were added (rabbit anti-  
140 mouse IgG [A-1902, Sigma Chemical Co.]; protein G [10-1222, Zymed Laboratories Inc.,  
141 San Francisco, CA]; goat anti-rat IgG [A-9654, Sigma Chemical Co.]; donkey anti-sheep  
142 IgG [A-5187, Sigma Chemical Co.]; rabbit anti-goat IgG [A-4187, Sigma Chemical Co.];  
143 goat anti-human IgG [2010-04, Southern Biotech. Assoc., Birmingham, AL]; goat anti-  
144 rabbit IgG [A-8025, Sigma Chemical Co.]; goat anti-guinea pig IgG [A-5062, Sigma  
145 Chemical Co.]; rabbit anti-horse IgG [A-6167, Sigma Chemical Co.]; rabbit anti-bovine  
146 IgG [A-0705, Sigma Chemical Co.]; mouse anti-black rhino [HL1530, Hybridoma Core  
147 Laboratory], and chicken anti-manatee IgG [9892, Hybridoma Core Laboratory]) at  
148 dilutions of 1:1000 and 1:2000 in PBS-AZ for each. All wells were done in duplicate.  
149 Plates were incubated at room temperature for 1 h. After incubation and washing, each well  
150 received 0.1 ml of alkaline phosphatase substrate (1 mg/ml *p*-nitrophenyl phosphate,  
151 Sigma Chemical Co.). Color development was monitored visually, and the absorbance at  
152 405 nm ( $\text{OD}_{405}$ ) was recorded after 30 and 60 min by use of a microplate reader  
153 (Spectramax 250, Molecular Devices, Sunnyvale, CA).

## 154 2.3. Purification of flying fox immunoglobulin

155  
156 Purification of *P. hypomelanus* immunoglobulin was performed using a commercial  
157 protein G column (HiTrap Protein G HP 5 ml, GE Healthcare, Piscataway, NJ). The  
158 column was prewashed with 50 ml PBS-AZ. A 0.5 ml aliquot of *P. hypomelanus* plasma  
was diluted 1:5 in PBS-AZ and circulated over the column for 50 min. The column was  
washed with 65 ml of PBS-AZ. IgG was eluted from the column using 0.1 M glycine, pH

159  
160 3.0. Fractions were collected in 3.0 ml aliquots as they eluted from the column.  
161 Absorbance at 280 nm of each fraction was measured, and peak fractions were pooled. The  
162 eluate was desalted using a centrifugal filter device (Ultrafree, Millipore, Billerica, MA  
163 01821) with a 30 kDa cut-off. The eluted protein was electrophoresed (2100 Bioanalyzer,  
164 Agilent Technologies, Palo Alto, CA) and measured.

#### 165 2.4. Polyclonal antibody production

166 Purified *P. hypomelanus* IgG was delivered to a private biological company (Strategic  
167 Biosolutions, Newark, DE) for polyclonal antiserum production. Two rabbits were  
168 immunized using their standard protocol. Plasma samples were obtained from each rabbit  
169 before immunization (preimmune plasma). Rabbits were immunized with 200 µg of *P.*  
170 *hypomelanus* IgG with complete Freund's adjuvant (first injection), then with incomplete  
171 Freund's adjuvant (all subsequent injections). Inoculations were given on days 0, 21, 35,  
172 and 49, and plasma samples were obtained on days 42, 57 and 63. Rabbits were  
173 anesthetized and exsanguinated on day 89. Serum samples were stored at  $-70^{\circ}\text{C}$ .

#### 174 2.5. Monoclonal antibody production

175 Mouse monoclonal antibodies against *P. hypomelanus* IgG were produced by standard  
176 protocols used by the Interdisciplinary Center for Biotechnology Research Hybridoma  
177 Core Laboratory at the University of Florida [17]. Briefly, a Balb/c female mouse was  
178 immunized subcutaneously with 30 µg of *P. hypomelanus* IgG in MPL + TDM adjuvant.  
179 Immunization was repeated on days 17, 92, and 143. Serum samples were obtained from  
180 the tail vein on days 27 and 103. The mouse was euthanized and the spleen was harvested.  
181 Spleen cells from the immunized mice were fused with Sp2/0 mouse myeloma cells at a  
182 ratio of 7:1 using 50% polyethylene glycol. Supernatants from the resulting hypoxanthine/  
183 aminopterin/thymidine (HAT) resistant hybridoma cells were evaluated for the presence  
184 of antibody that bound to *P. hypomelanus* IgG by ELISA using methods similar to those  
185 described above, with *P. hypomelanus* IgG-coated wells and alkaline phosphatase labeled  
186 rabbit anti-mouse IgG (A-1902) as a secondary antibody. Hybridomas from wells with  
187 positive antibody results ( $\text{OD}_{405}$  3–20 $\times$  over background) were transferred to 24 well  
188 plates and screened by an ELISA a second time. One hybridoma with positive antibody  
189 results was selected and cloned. The cloned hybridoma cell line was designated HL1892.

#### 190 2.6. Detection of the humoral immune response

191 ELISAs were used to monitor the humoral immune response to DNP-BSA in variable  
192 flying foxes. Wells of a high protein binding microplate (Nunc Maxisorp, Fisher Scientific)  
193 were coated with 50 µl of DNP-BSA (1.0 µg/ml) and were left to adsorb overnight at  $4^{\circ}\text{C}$ .  
194 After this and each subsequent step, all wells were washed three times with PBS-AZ and  
195 0.05% Tween-20 using an automated microplate washer. After washing, all wells were  
196 blocked with PBS-AZ containing 5% nonfat dry milk (NFDM). This and each subsequent  
197 step of the ELISA were incubated with gentle agitation for 1 h at approximately  $25^{\circ}\text{C}$ .  
Variable flying fox plasma samples were diluted from 1:25 to 1:32,000, and replicate wells

198 were done for each dilution. PBS-AZ was applied to a pair of wells for each plasma sample  
199 to serve as duplicate negative control wells. Fifty microliter of preimmune and immune bat  
200 plasma dilutions were used to coat the wells. Alkaline phosphatase-conjugated protein G  
201 (1:500 in PBS-AZ), rabbit anti-*P. hypomelanus* IgG polyclonal antibody (1:10,000 in PBS-  
202 AZ), and mouse anti-*P. hypomelanus* IgG monoclonal antibody (undiluted hybridoma  
203 supernatant) were added for the detection of bound bat antibodies. Alkaline phosphatase-  
204 conjugated goat anti-rabbit IgG (Sigma A-8025, 1:2000 in PBS-AZ) and rabbit anti-mouse  
205 IgG (A-1902, 1:1000 in PBS-AZ) were used as secondary antibodies for polyclonal or  
206 monoclonal antibodies, respectively. After washing, 0.1 ml of 1 mg/ml *p*-nitrophenyl  
207 phosphate was added for detection. Color development was monitored visually, and the  
208 OD<sub>405</sub> was recorded after 30 and 60 min by use of a microplate reader. For analysis, the  
209 average OD<sub>405</sub> of the negative controls were subtracted from the average OD<sub>405</sub> readings of  
210 all other wells of the corresponding antibody (corrected OD<sub>405</sub>).  
211

### 212 2.7. Western blot analysis of polyclonal and monoclonal antibodies

213 For further evaluation of the specificity of the polyclonal and monoclonal antibodies,  
214 variable flying fox plasma was separated by gel electrophoresis in SDS-PAGE under  
215 reducing conditions using pre-cast 10% Bis-Tris gels (Invitrogen, San Diego, CA) and then  
216 electrophoretically transferred from the gel to a nitrocellulose membrane (Invitrogen). A  
217 Tris-glycine buffer (Invitrogen) in 20% methanol was used as the transfer buffer. The  
218 blotting time was 60 min at 30 V. After transfer, the nitrocellulose was immediately  
219 blocked overnight with PBS-AZ containing 5% NFDM at room temperature  
220 (approximately 25 °C). The nitrocellulose blot was washed three times for 5 min each  
221 with PBS solution containing 0.02% sodium azide and 0.05% Tween-20 and placed into a  
222 channel divider (Fast Blot Developer, Pierce, Rockford, IL). The rabbit polyclonal antisera  
223 was diluted 1:50,000. A 1:50,000 dilution of rabbit preimmune sera was used as a negative  
224 control. A total of 900 µl of primary antibody (polyclonal or monoclonal antibodies) was  
225 loaded into each channel and incubated on the nitrocellulose for 60 min at room  
226 temperature on a rocker. After washing, the nitrocellulose was removed from the  
227 manifold and incubated with the appropriate secondary antibody: alkaline phosphatase  
228 labeled goat anti-rabbit IgG (diluted 1:2000 in PBS-AZ) or goat anti-mouse IgG (diluted  
229 1:4000 in PBS-AZ) for the polyclonal antibody and monoclonal antibodies, respectively,  
230 for 60 min. After washing, the blot was developed with nitroblue tetrazolium 5-bromo-4-  
231 chloro-3 inolyphosphate *p*-toluidine substrate, per manufacturer's instructions (Sigma  
232 Chemical Co.).

### 233 2.8. Cross-reactivity of rabbit polyclonal and mouse monoclonal anti-*P. hypomelanus* 234 IgG antibodies

235 The ability of the rabbit polyclonal antibody and monoclonal antibody HL1892 to cross-  
236 react with previously banked plasma from eight bat species, including six other species in  
237 the genus *Pteropus* (*P. conspicillatus*, *P. giganteus*, *P. poliocephalus*, *P. pumilus*, *P.*  
238 *rodricensis*, and *P. vampyrus*), another megachiropteran species (*Eidolon helvum*), and a  
phyllostomatid microchiropteran (*Phyllostomus hastatus*), was evaluated by an ELISA as

Table 1

Corrected OD<sub>405</sub> values for ELISAs using polyclonal rabbit anti-*P. hypomelanus* IgG and monoclonal mouse anti-*P. hypomelanus* IgG for detection

	Polyclonal	Monoclonal
<i>Pteropus hypomelanus</i>	3.666	0.755
<i>Pteropus conspicillatus</i>	2.84	0.3935
<i>Pteropus rodricensis</i>	2.5865	0.3735
<i>Pteropus poliocephalus</i>	3.3095	0.8675
<i>Pteropus giganteus</i>	2.649	0.5355
<i>Pteropus pumilus</i>	3.234	0.7655
<i>Pteropus vampyrus</i>	3.0525	0.4165
<i>Eidolon helvum</i>	2.025	0.0105
<i>Phyllostomus hastatus</i>	2.0765	−0.006

Six different species in the genus *Pteropus* are represented. *E. helvum* is a megachiropteran outside the genus *Pteropus*. *P. hastatus* is a microchiropteran.

described above. Wells were coated with 50 µl of a 1:100 dilution of plasma samples from each of the eight bat species. Fifty microliter of a 1:5000 dilution of rabbit polyclonal anti-*P. hypomelanus* IgG or undiluted mouse monoclonal anti-*P. hypomelanus* IgG hybridoma supernatant was then evaluated for reactivity on these plasma samples. A 1:5000 dilution of rabbit preimmune sera was used as a negative control for the polyclonal antibody ELISA, and hybridoma culture media was used as a negative control for the monoclonal antibody ELISA. Appropriate secondary antibodies were used as described above. The polyclonal antibody ELISA was read at 30 min and the monoclonal antibody ELISA was read at 60 min. For analysis, the average OD<sub>405</sub> of the negative controls were subtracted from the average OD<sub>405</sub> readings of all other wells of the corresponding antibody (corrected OD<sub>405</sub>). Specificity of this reaction was confirmed by western blot analysis as described above using serum from the different bat species (Table 1).

### 3. Results

#### 3.1. Cross-reactivity of other anti-immunoglobulin reagents with *P. hypomelanus* IgG using ELISA

At 60 min, a 33-fold increase in absorbance over the negative control was seen using a 1:1000 dilution of alkaline phosphatase-conjugated protein G. At 60 min, a 10-fold increase over the negative control was seen using a 1:1000 dilution of goat anti-human IgG-lambda chain specific. These wells were detected with a rabbit anti-goat IgG alkaline phosphatase conjugate. Significant increase was not seen with other anti-immunoglobulin antibodies. A protein G column was therefore used for purification of *P. hypomelanus* IgG.

#### 3.2. Purification of *P. hypomelanus* immunoglobulin

The protein eluted from the protein G column measured 161 kDa unreduced and had two bands of 58.1 and 28.7 kDa when reduced (Fig. 1). This was comparable in size to

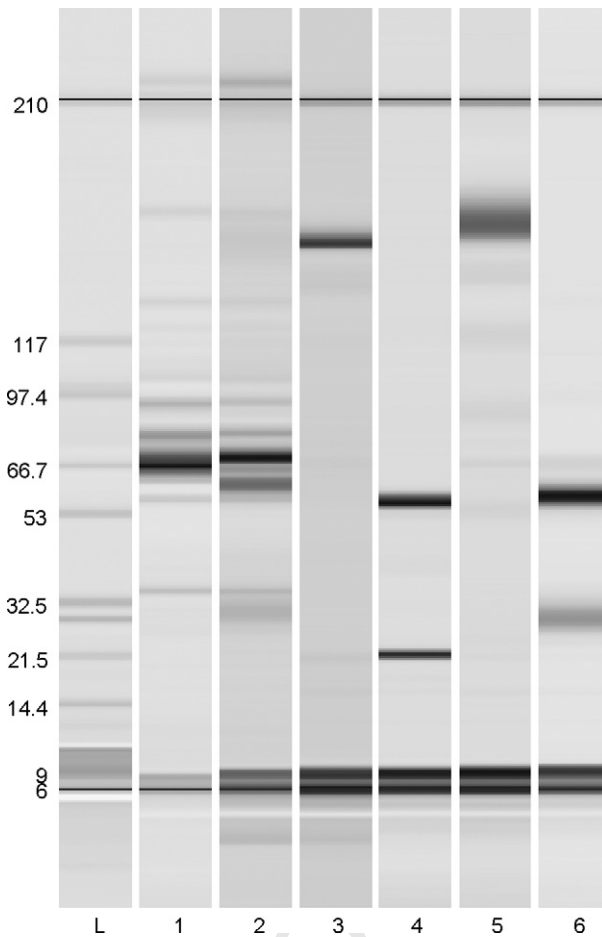


Fig. 1. Protein electrophoresis to assess purified *P. hypomelanus* IgG. L, ladder. Lane 1, unpurified *P. hypomelanus* plasma. Lane 2, unpurified *P. hypomelanus* plasma. Lane 3, unreduced mouse IgG. Lane 4, reduced mouse IgG. Lane 5, unreduced *P. hypomelanus* IgG. Lane 6, reduced *P. hypomelanus* IgG.

mouse IgG, which measured 154.9 kDa unreduced and had two bands of 56.4 and 21.5 kDa when reduced on the same equipment. In other reports, bat IgGs were comparably sized [18,19].

### 3.3. Detection of the humoral immune response

Immunized variable flying foxes developed an anti-DNP-BSA titer compared with preimmune plasma. Use of protein G in an ELISA showed the greatest increase in antibody titer between pre-vaccination and 56 day post-vaccination variable flying fox plasma with samples diluted 1:2000 in PBS (Fig. 2). Detection of the anti-DNP-BSA antibody titer by use of the polyclonal rabbit anti-*P. hypomelanus* IgG antibody showed the greatest increase in increase in antibody titer between pre-vaccination and 56 day post-vaccination variable

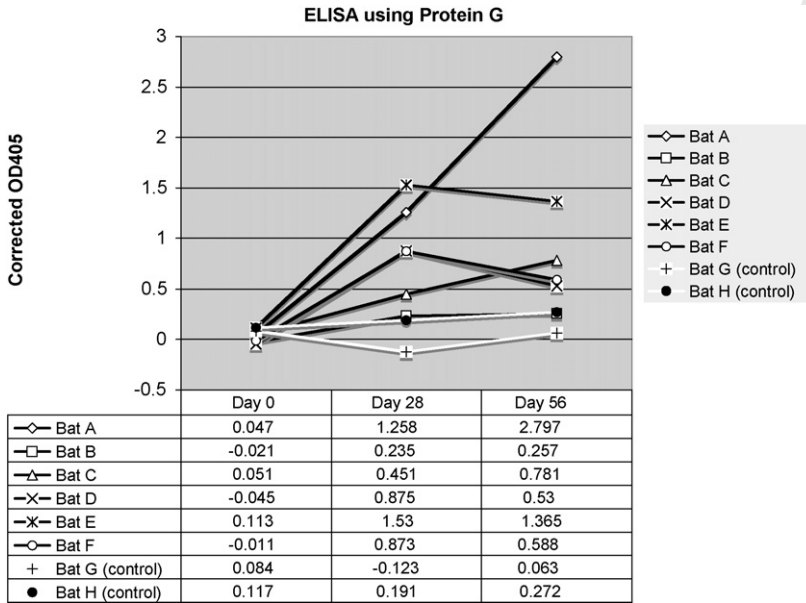


Fig. 2. Corrected OD<sub>405</sub> values for an ELISA using protein G for detection. Plasma was diluted 1:2000. Vaccinated bats are represented by black lines, and control bats are represented by white lines.

flying fox plasma samples diluted 1:800 (Fig. 3). Detection of the anti-DNP-BSA antibody titer by use of the monoclonal mouse anti-*P. hypomelanus* IgG antibody HL1892 showed the greatest increase in antibody titer between pre-vaccination and 56 day post-vaccination variable flying fox plasma samples diluted 1:100 (Fig. 4). Bats A and E consistently showed strong antibody responses to DNP-BSA in all assays, whereas bat B showed little to no antibody response to vaccination.

### 3.4. Western blot analysis of polyclonal and monoclonal antibodies

Western blot reactivity of anti-*P. hypomelanus* IgG's on variable flying fox plasma was determined (Fig. 5). The polyclonal antibody (lane 1) reacted with major bands at approximately 58 and 29 kDa, consistent with IgG heavy chain and light chain, respectively. Multiple weaker bands ranging from >191 to 15 kDa were also seen, and were interpreted as non-specific binding. Monoclonal antibody HL1892 (lane 2) reacted with a band at approximately 58 kDa, consistent with IgG heavy chain. Non-specific binding was not seen under the conditions used. No reaction was seen with the negative control (lane 3).

### 3.5. Cross-reactivity of rabbit polyclonal and mouse monoclonal anti-*P. hypomelanus* IgG antibodies

The polyclonal anti-*P. hypomelanus* IgG antibody was found to cross-react well against plasma of all bat species tested by ELISA, although more strongly with bats in the genus

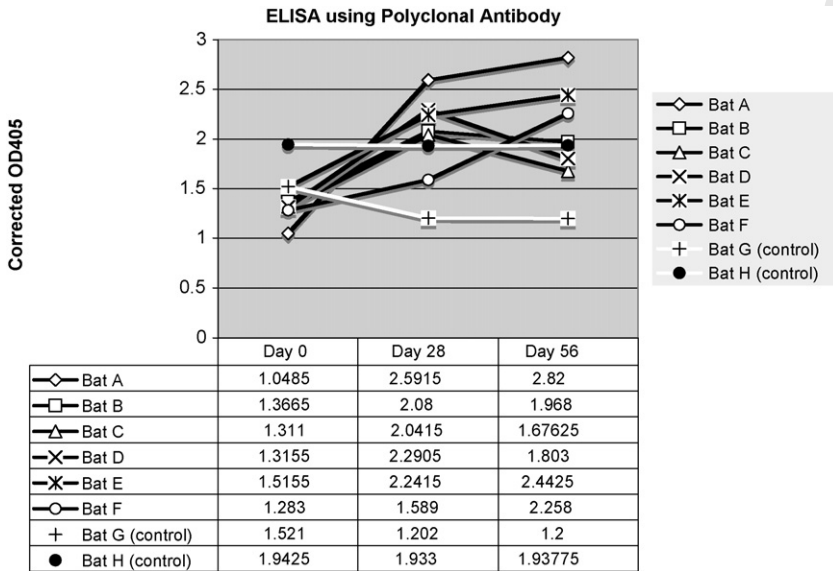


Fig. 3. Corrected OD<sub>405</sub> values for an ELISA using polyclonal rabbit anti-bat IgG antibody for detection. Plasma was diluted 1:800. Vaccinated bats are represented by black lines, and control bats are represented by white lines.

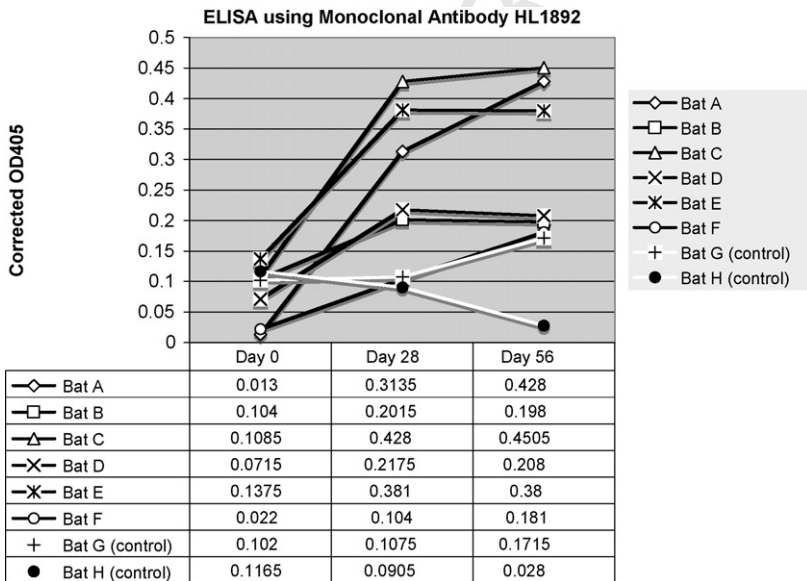


Fig. 4. Corrected OD<sub>405</sub> values for an ELISA using monoclonal mouse anti-bat antibody HL1892 for detection. Plasma was diluted 1:100. Vaccinated bats are represented by black lines, and control bats are represented by white lines.

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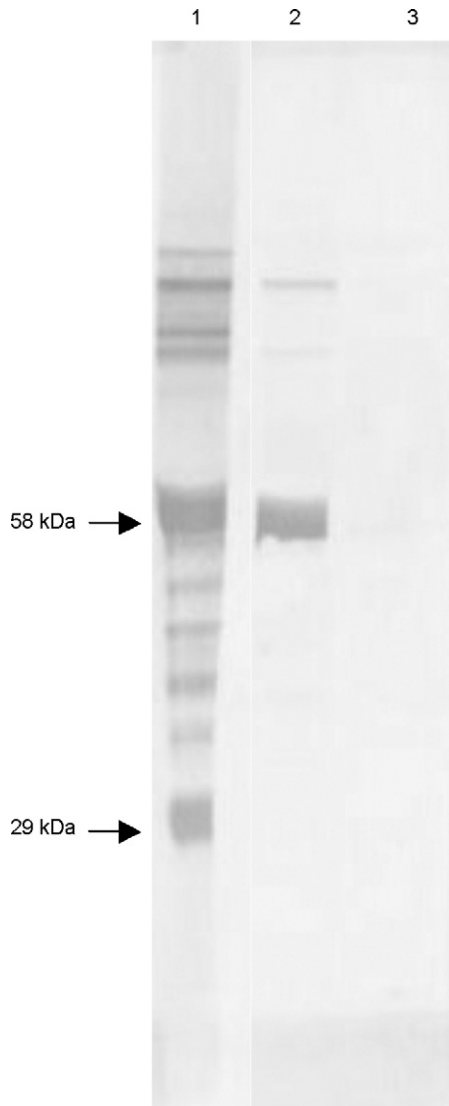


Fig. 5. Western blot reactivity of anti-*P. hypomelanus* IgG's on variable flying fox plasma. Lane 1, polyclonal rabbit anti-bat antibody. Lane 2, monoclonal mouse anti-bat antibody HL1892. Lane 3, preimmune rabbit serum (negative control).

*Pteropus*. The monoclonal antibody HL1892 was found to cross-react by ELISA equally as well against plasma of *P. poliocephalus* and *P. pumilus* as it did against *P. hypomelanus* plasma. Strong reaction was also seen with plasma from other members of the genus *Pteropus* tested, with OD<sub>405</sub> readings 49–70% of those seen against *P. hypomelanus* plasma. OD<sub>405</sub> readings against plasma from *E. helvum* or *P. hastatus* were not significantly different from that seen with negative controls. On western blot using the anti-*P.*

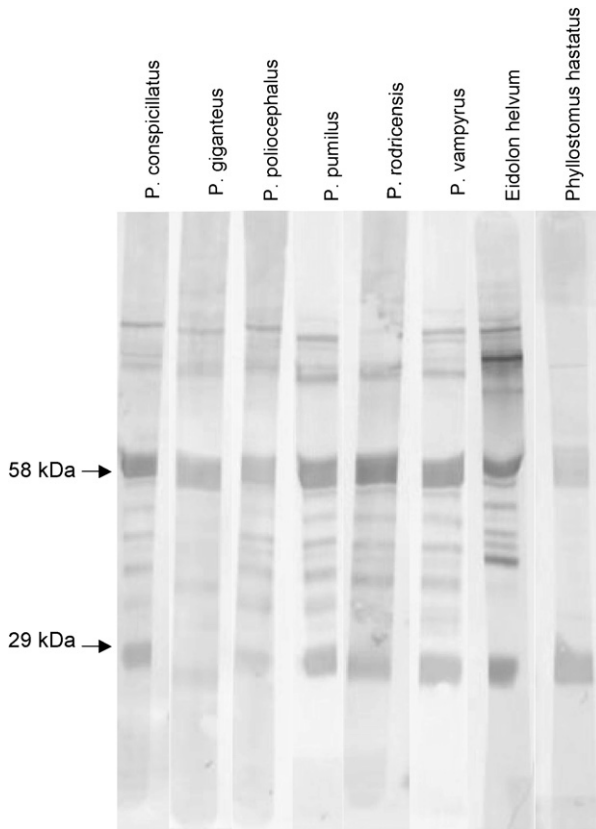


Fig. 6. Reactivity of polyclonal rabbit anti-bat antibody against sera from eight different bat species. Six different species in the genus *Pteropus* are represented. *Eidolon helvum* is a megachiropteran outside the genus *Pteropus*. *Phyllostomus hastatus* is a microchiropteran.

*hypomelanus* IgG polyclonal antibody for detection, major bands were detected at approximately 58 and 29 kDa with all bat plasma tested, consistent with IgG heavy chain and light chain, respectively (Fig. 6). Cross-reaction with some non-specific background similar to that seen with *P. hypomelanus* plasma was seen for all bat sera tested. Monoclonal antibody HL1892 (Fig. 7) reacted with a band at approximately 58 kDa in all bat plasma, consistent with IgG heavy chain. Non-specific binding was not seen under the conditions used. No reaction was seen with the negative control (data not shown).

#### 4. Discussion

The reagents used in this study may be useful for the development of ELISA and western blot tests for antibodies to pathogens found in *Pteropus* spp. Although neutralization assays are accepted as the reference standard [11], there are several

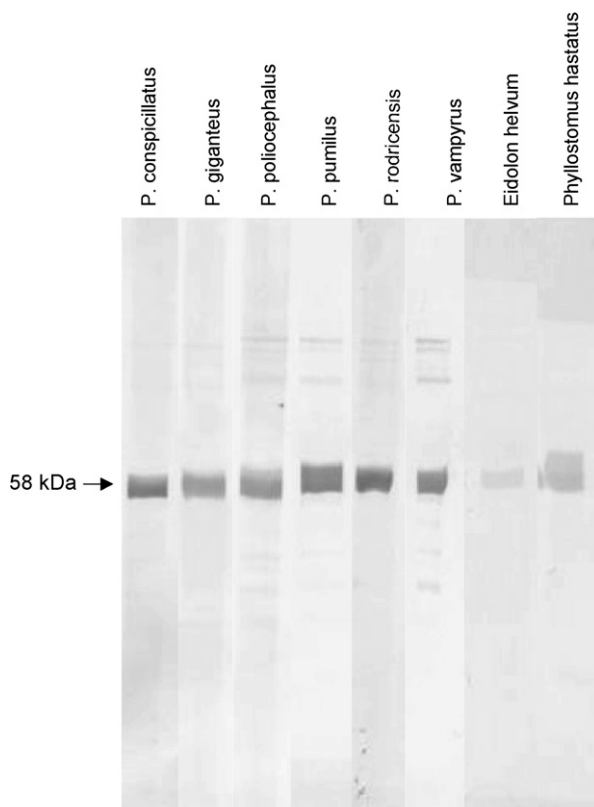


Fig. 7. Reactivity of monoclonal mouse anti-bat antibody HL1892 against sera from eight different bat species. Six different species in the genus *Pteropus* are represented. *Eidolon helvum* is a megachiropteran outside the genus *Pteropus*. *Phyllostomus hastatus* is a microchiropteran.

disadvantages to these assays. Serum neutralization requires a period of days to weeks to obtain results, and early diagnosis of infection may be important for epidemiologic control. Serum neutralization assays also require pathogen culture, and as some emerging infections of bats represent serious biohazards, the risk to personnel is significant, and appropriate facilities for culture are limited. Serum neutralization also needs consistent CPE, and some viruses may have limited CPE.

In addition to serum neutralization, several other methods have been used for the detection of antibodies in bats, including hemagglutination inhibition [20], complement fixation [21], and gel immunoprecipitation [22].

Early work showed that in big brown bats (*Eptesicus fuscus*) experimentally infected with Japanese encephalitis virus, the predominant antibody type shifted from a heavier protein (corresponding to IgM) to a lighter protein (corresponding to IgG) within 20 days [23]. A study using bacteriophage  $\phi$ X174 as an antigen in big brown bats found that vaccinated animals had converted to an IgG response by 8 weeks [24]. Bats had high

neutralizing antibody titers 3.5 years after vaccination. Great fruit-eating bats (*Artibeus lituratus*) were shown to develop strong IgM and IgA responses to experimental *Histoplasma capsulatum* infection at 3–5 weeks and IgG responses by 8–9 weeks [22]. Within the genus *Pteropus*, Indian flying foxes (*P. giganteus*) immunized with sheep erythrocytes had a maximal IgG response at 15–30 days, depending on immunization dose [25]. Vampire bats (*Desmodus rotundus*) orally vaccinated with a recombinant rabies vaccine showed the protection 18 days post-vaccination, with maximal protection 30 days post-vaccination [26]. Protection decreased at 90 and 120 days post-vaccination. Egyptian fruit bats (*Rousettus aegyptiacus*) vaccinated with a killed rabies vaccine developed neutralizing antibody titers [27].

Previous ELISA assays have been used in bats. ELISAs using a chimeric protein A/G as conjugate have been used for the detection of antibodies to Nipah and Hendra viruses in bats, and an ELISA using this protocol has been found to have a specificity of 98.4% for Nipah virus in pigs [11]. A similar ELISA was used to screen for lyssavirus in Cambodian bats [12]. In the lyssavirus study, ELISA-positive sera were confirmed using a neutralization assay, and only 27% had neutralizing antibodies. Reasons for this discrepancy may be either low specificity of the lyssavirus ELISA, low sensitivity of the neutralization assay, or detection of antibodies to non-neutralizing epitopes.

ELISA methods that do not require binding of the constant region of immunoglobulins have also been developed. These assays require pathogen-specific rather than host antibody-specific reagents. Competitive ELISA methods have been used to detect antibodies to Nipah virus in lesser short-nosed fruit bats (*Cynopterus brachyotis*) and common dawn bats (*Eonycteris spelaea*), two megachiropteran species [28]. A capture ELISA has been used for detection of Nipah and Hendra virus-specific IgM [11].

It is important to remember that antibody assays only measure one aspect of the immune system, and cellular immune responses are likely to be more important for intracellular pathogens such as viruses. In a study on rabies vaccination in vampire bats, 31 of 32 bats were protected from challenge by vaccination, whereas 9 of 10 unvaccinated bats succumbed [29]. Of these 31 protected bats, 9 (29%) did not develop a neutralizing antibody titer, implying a large role for cellular immunity in protection. Absence of antibody does not demonstrate that an animal has not been exposed to a pathogen.

Control strategies for dealing with bat-borne diseases have primarily been centered on elimination of bat populations. This is unacceptable when applied to endangered species, and is likely to be less effective than vaccination strategies [29,30]. There are currently no active immunization programs in wild bats [31]. Bats are fastidious groomers, and vampire bats have been shown to take up anticoagulants applied to the backs of conspecifics [26]. Oral rabies vaccines have been shown to be effective against challenge in vampire bats [26,29,32]. Oral subunit vaccines delivered via transgenic plants have been suggested [31].

Polyclonal antibodies to IgG of another megachiropteran species, the Egyptian fruit bat (*Rousettus aegyptiacus*), have been made [18]. Based on their western blot results, their polyclonal antibodies appear to have a greater specificity for chiropteran IgG than the more broadly cross-reactive polyclonal antibodies developed in this study. Their antibodies were used to determine phylogeny by examining cross-reactivity with other species. This may be a future use for the antibodies developed in this study. Evidence is mounting that the phylogenetic division between the suborders Microchiroptera and Megachiroptera is a

368 false dichotomy, and that microchiropterans are not monophyletic. Molecular sequence  
369 data suggests that Rhinolophoid bats actually cluster with Pteropodid bats, which were  
370 considered the sole family in the megachiroptera [1]. These bats have been classified into  
371 the Yinpterochiroptera, and the remaining microchiropterans classified into the  
372 Yangochiroptera. No Rhinolophoid bat sera were examined, so no conclusions can be  
373 drawn regarding cross-reactivity, but this may be a direction for further studies.

374 In conclusion, protein G, polyclonal anti-*P. hypomelanus* IgG, and monoclonal anti-*P.*  
375 *hypomelanus* IgG all detected specific anti-DNP-BSA antibody responses in immunized  
376 variable flying foxes. The polyclonal antibody was found to cross-react well against IgG of  
377 all bat species tested, but did not show as great a difference between vaccinated and  
378 unvaccinated bats. This was likely due to non-specific binding as seen on the western blot,  
379 resulting in high backgrounds. The monoclonal antibody was found to cross-react well  
380 against IgG of six other species in the genus *Pteropus* and to cross-react less strongly  
381 against IgG from *E. helvum* or *P. hastatus*. Protein G distinguished best between vaccinated  
382 and unvaccinated bats, and these results validate the use of protein G for detection of bat  
383 IgG antibodies. The monoclonal antibodies developed in this study recognized  
384 immunoglobulins from other members of the genus *Pteropus* well, and may be useful  
385 in applications where specific detection of *Pteropus* IgG is needed. Further investigation of  
386 these reagents in diagnostic assays is merited.  
387

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