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1 **IgY-based antivenom against *Bothrops alternatus*: production and neutralization efficacy**

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28 **Abstract**

29 Antivenom for the treatment of bothropic snakebite is a priority for public health institutions
30 from Latin America. An alternative to the conventional antivenom production is based on the use of
31 egg yolk antibodies – IgY-technology – by immunizing laying hens. In this study, we produced,
32 characterized and assessed the efficacy of IgY-based antivenoms against *B. alternatus* venom.
33 Immunochemical studies (reactivity, avidity and antigen recognition pattern) as well as antivenom
34 efficacy assays were performed. After the 3rd immunization, levels of specific IgY reached a
35 maximum that was maintained throughout the observation period, while avidity indexes of the
36 extracts increased after the successive immunizations. Furthermore, IgY against *B. alternatus*
37 recognized protein complexes of the venom with high (> 40 kDa), medium (20-40 kDa) and low (<
38 20 kDa) molecular weights. IgY antivenoms obtained after 8 immunizations neutralized 35.65 µg of
39 *B. alternatus* venom per mg of antivenom, while specific activities values ranged from 0.28 to 0.42.
40 In conclusion, we produced and characterized IgY antivenoms capable of neutralizing the lethal
41 activity of *B. alternatus* venom at a preclinical level. Thus, IgY-technology may allow the
42 production of effective and affordable antivenoms fulfilling the urgent needs of many countries
43 where conventional manufacture is unable to provide enough availability of antivenoms.

44

45 **Keywords**
46 *Bothrops alternatus*, snake venom, IgY antibody, egg yolk, snakebite, venom neutralization.

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54 **1. Introduction**

55

56 Snakebite envenoming is a neglected tropical disease that has a significant impact on Public
57 Health. Five million snakebites leading to 400.000 disabilities and 138.000 deaths per year are
58 estimated to occur worldwide (Gutiérrez *et al*., 2014; World Health Organization, 2017), although
59 its true incidence is unknown (Gutiérrez *et al*., 2017a; Kasturiratne *et al*., 2008). In Latin America,
60 snakebite envenoming runs from 80,329 to 129,084 cases per year with a high estimate of 2,298
61 deaths (Kasturiratne *et al*., 2008). In South America, *Bothrops alternatus* (Viperidae family) is a
62 species involved in snakebite envenomings due to its widespread distribution in Argentina, Brazil,
63 Paraguay and Uruguay (Gutiérrez, 2011; World Health Organization, 2017). This disease has a high
64 incidence in places where provision of health service is not efficient enough such as occurring in
65 rural areas (Gutiérrez *et al*., 2017b; World Health Organization, 2017). Accidental envenoming by
66 *Bothrops* spp. usually causes severe tissue damage around the bite site, inducing edema,
67 inflammation, hemorrhage and myonecrosis. Systemic failures such as blood incoagulability and
68 thrombocytopenia may also occur (León *et al*., 2011; Sousa *et al*., 2013), though renal failure is the
69 principal cause of death in human patients (de Roodt *et al*., 1997; Queiroz *et al*., 2008). Bothropic
70 venom is composed by around 100 different peptides and its pathogenesis is mainly due to the
71 presence of metallo- and serine proteinases (svMPs and svSPs) (Öhler *et al*., 2010; Queiroz *et al*.,
72 2008).

73 Antivenoms are considered essential medicines for treating snakebite envenomings.
74 Conventional production is based on the immunization of large animals, mainly horses, with
75 mixtures of representative venoms of a determined geographical area. The hyperimmune plasma
76 obtained after immunization is followed by fractionation methods (Gutiérrez *et al*., 2017a; Segura *et*
77 *al*., 2010). In order to guarantee a good quality of the antivenom, purification steps and control of
78 infectious risks are mandatory assessments (Dos-Santos *et al*., 2011; World Health Organization,
79 2017). Nevertheless, equine-derived antivenoms usually induce dose-related early and delayed

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80 anaphylactic reactions in patients, such as serum sickness which produce vasculitis,
81 glomerulonephritis and arthritis (Laustsen *et al*., 2018a; Otero-Patiño *et al*., 2012). On the other
82 hand, antivenom production still remains a high-cost process leading to a global ongoing reduction
83 in the manufacturers (Alirol *et al*., 2015; Fry *et al*., 2003; Navarro *et al*., 2016; World Health
84 Organization, 2017). Since snakebite envenoming remains a global health issue and has been
85 recently included into Category A of Neglected Tropical Diseases by the World Health
86 Organization, great efforts are needed to provide availability of these complex biological medicines
87 in the primary health-care system (Chippaux and Goyffon, 1991; Chippaux, 2017).

88 The use of egg yolk antibodies – IgY-technology – represents an alternative to the
89 conventional antivenom production due to its economical, ethical and productive advantages
90 (Chacana *et al*., 2004; Theakston *et al*., 2003). In Costa Rica, Navarro *et al*. (2016) compared the
91 animal maintenance costs, procedures and supplies needed to keep horses and hens showing that the
92 production prime cost can be reduced around a 40 %. Also, horses required for obtaining
93 antivenoms should be between 3 and 10 years old (World Health Organization, 2017) while hens
94 start laying eggs at ~20 weeks of age (Yuan *et al*., 2015). This fact, together with the
95 industrialization of poultry production worldwide, may reduce maintenance costs of the animals and
96 facilitate the provision and replacement of hens to obtain the immunoglobulins.

97 Furthermore, sampling is non-invasive since the bleeding of the animal is replaced by egg
98 collection, and therefore pain and distress of animals are sensibly reduced (Gruber and Hartung,
99 2004). In addition, the present lines of laying hens are able to produce between 17 and 35 g of IgY
100 per year of which 1-10 % is antigen-specific (Pauly *et al*., 2011; Schade *et al*., 2005). Diversity of
101 methods used to determine the neutralization efficacy as well as the intrinsic complexity of the
102 venoms make very difficult to undergo a comparative analysis to assess the feasibility of IgY-
103 technology as an alternative to the production in horses (Lanari *et al*., 2014; Segura *et al*., 2013).
104 However, several preclinical testing of IgY-based antivenoms have been reported with promising

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105 results (da Rocha *et al*., 2017; de Almeida *et al*., 2008; Duan *et al*., 2016; Lee *et al*., 2016b;
106 Meenatchisundaram *et al*., 2008).

107 Because the production of specific antidotes for the treatment of common bothropic snake
108 bites has a high priority in public health institutions in Latin American (Gutiérrez *et al*., 2009;
109 World Health Organization, 2017), we produced and characterized an IgY-based antivenom against
110 *B. alternatus* and evaluated its efficacy in mice.

111

112 **2. Materials and Methods**

113

114 **2.1. Ethical statement**

115 This study meets the ARRIVE guidelines (Kilkenny *et al*., 2010). The experiments were
116 approved by the Institutional Animal Care and Use Committee (IACUC) from the CICVyA-INTA,
117 Procedure #20/2012.

118

119 **2.2. *B. alternatus* venom**

120 Freeze-dried mixture of *B. alternatus* venoms were obtained from adult specimens that were
121 provided by the National Administration of Laboratories and Institutes of Health (ANLIS) "Dr.
122 Carlos G. Malbrán" from Argentina. Previously, it was determined that the batch of venom used in
123 this study has a median lethal dose (LD_{50}) of 28.28 µg, 56.57 µg and 200 µg per mouse by
124 intravenous (i.v.), intraperitoneal (i.p.) and intramuscular (i.m.) routes, respectively (unpublished
125 data).

126

127 **2.3. Animals**

128 Four Lohmann Brown laying hens of 16 weeks were housed into individual cages. NIH mice
129 of 18-22 g were provided by the Central Bioterium (ANLIS); mice were housed into plastic boxes.

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130 Mice and hens were given water and feed *ad libitum* and maintained with cycles of light/ dark of
131 12/12 h and 14/10 h, respectively.
132

133 **2.3.1. Hens immunization**

134 Hens were intramuscularly injected with *B. alternatus* whole venom into their breasts. A
135 two-cycle inoculation scheme was considered. For all first double doses (subcutaneous under the
136 skin behind the neck and intramuscular into the breast muscles) the venom was emulsified in
137 Freund's complete adjuvant (FCA) whereas for subsequent intramuscular booster doses Freund's
138 incomplete adjuvant (FIA) was used. The first cycle consisted in 3 immunizations each 15 days,
139 injecting increasing amounts of venom (400 μg, 800 μg and 1200 μg); 2 months after the third
140 injection hens were not immunized anymore (period of rest). The second cycle consisted in 5
141 immunizations also separated by 15 days and hens were inoculated with different amounts of
142 venom (Table 1). Serum samples were taken 7 days after each immunization. Eggs were collected
143 during 10 days after the 3rd, 4th and 8th immunizations.
144

145 **2.4 IgY purification**

146 IgY from eggs collected from each hen was extracted according to the procedure described
147 by Akita and Nakai (1992). Accordingly, double-precipitation with ammonium sulphate (24 and 26
148 % w/v) was used to purify IgY. Extracts were dialyzed against saline solution and 0.01 % w/v
149 thimerosal was added to avoid microbial contamination. Sulphate traces in the extracts were
150 detected with barium chloride as described by Laborde *et al*. (1989). The total protein content of
151 IgY extracts was determined by a Bradford standard procedure for microtiter plates using Bio-Rad
152 protein reagent and bovine serum albumin (Sigma-Aldrich) as standard. Purified IgY was kept at
153 4°C until use.

154

155 **2.5 Immunochemical studies**

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157 **2.5.1 Solid-phase enzyme immunoassay (ELISA)**

158 Maxisorp microtiter plates (Thermo Scientific) were coated with 100 µL of a solution of *B.*
159 *alternatus* venom (2 µg/ well) in 100 mM sodium carbonate buffer (pH 9.5). After overnight (ON)
160 incubation at 4°C, the plates were washed three times with PBS plus 0.05 % Tween (PBS-T) and
161 100 µL of blocking buffer (PBS-T plus 5 % skim milk) were added to each well. After incubation
162 for 1 h at 37ºC, the wells were washed as described above and filled with 100 µl of 2-fold serially
163 diluted IgY antivenom or chicken sera. The plates were incubated for 1 h at 37°C and washed three
164 times. Afterwards, 100 µL of rabbit anti-IgY antibodies conjugated with peroxidase (Catalogue #
165 A9046; Sigma-Aldrich) diluted 1:5000 in PBS-T were added to each well and incubated. After a
166 final washing step, color was developed by the addition of ABTS (Catalogue # P9029; Sigma-
167 Aldrich), 50 mM citrate solution (pH 4.2) and 3 % H_2O_2. Color development was stopped by adding
168 5 % SDS and absorbance at 405 nm was measured. The relative levels of antibody in the sample
169 were determined by calculating the sample to positive (S/P) ratio. In addition, an internal reference
170 (IgY specific for *B. alternatus* from a previously immunized hen) was included in each plate to
171 assess variance between assays.

172

173 **2.5.2 Chaotropic ELISA**

174 An ELISA avidity test was carried out to evaluate the avidity of the IgY extracts as has been
175 previously described with modifications (Baudou *et al.*, 2017; Sampaio *et al.*, 2014). Microtiter
176 plates were coated and incubated as mentioned above. Samples were diluted to reach similar
177 specific O.D. (~ 0.4) and incubated for 1 h. Later, 100 µL of 6 M urea was added to half of the
178 plates and left to react during ten minutes. Afterwards, the plates were washed three times with
179 PBS-T and the secondary antibody was added as described before. Absorbances at 405 nm were
180 recorded. Avidity index were expressed in percentage (AI %) and determined by the ratio between
181 optical density values of samples treated with urea and the optical density of untreated samples.

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182

183 **2.6. SDS-PAGE and immunoblotting**

184 Venom and IgY samples were analyzed by SDS-PAGE under reducing and non-reducing
185 conditions (Laemmli, 1970). Venom samples were separated on a 15 % SDS-PAGE gel and IgY
186 extracts on 12 % gels, subsequently stained with Coomassie Brilliant Blue R-250 (ICN Biomedicals
187 Inc.). Detection of venom proteins by IgY extracts were carried out by Western blot according to
188 Towbin's method (Towbin *et al*., 1979). Briefly, the proteins that were previously separated by
189 electrophoresis were subsequently electroblotted onto nitrocellulose membranes using a Mini Trans-
190 Blot system (Bio-Rad) for 2 h at 30 mA. After blotting, membranes were blocked ON at 4°C with
191 blocking buffer. Membranes were incubated for 1 h at 4°C with a 1:1000 dilution of IgY anti-*B.*
192 *alternatus* in PBS-T. After three washes, membranes were incubated for 1 h with peroxidase-
193 conjugated rabbit anti-chicken IgY (Catalogue # A9046; Sigma-Aldrich) diluted 1:5000 in PBS-T.
194 After washing, membranes were incubated with chromogenic substrate solution (10 mg of DAB, 10
195 ml PBS and 6.4 µl of H_2O_2). To evaluate the physicochemical purity of IgY extracts, electrophoretic
196 patterns were analyzed with a Gel Doc XR+ System (Bio-Rad) and densitometric scanning of the
197 stained gels was performed with ImageLab software (Bio-Rad).

198

199 **2.7. Neutralization of hemorrhagic and necrotizing activity**

200 Minimal hemorrhagic dose (MHD) and minimal necrotizing dose (MND) of the venom
201 were determined according to Theakston and Reid (1983) and their neutralization by IgY were
202 assessed as described by Instituto Clodomiro Picado (2007). Groups of 4 mice (19 ± 2 g) were
203 intradermically injected with a mixture containing 10 MHD or 10 MND of the venom which was
204 previously incubated with different quantities of IgY antivenom (range: 0.92 – 7.37 mg) for 30 min
205 at 37ºC in a final volume of 1 mL. In addition, 2 groups of 4 mice each were injected either with the
206 untreated venom or with 0.15 M NaCl. To determine the neutralization of the hemorrhagic activity,
207 mice were euthanized after 2 h and the major perpendicular diameters of the hemorrhagic haloes in

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208 the skin were measured at the dermal face. Likewise, neutralization of the necrotizing activity was
209 determined after 72 h. Neutralization of the activities was expressed as the mass (mg) of antivenom
210 required to neutralize both 10 MHD and 10 MND of the venom.

211

212 **2.8. Neutralization of venom lethality by IgY antivenoms**

213 To assess the efficacy of IgY antivenoms, the median effective dose (ED_{50}) assay was
214 performed according to WHO guidelines (World Health Organization, 2017). Briefly, 3 LD_{50} (84.80
215 µg) of B. *alternatus* venom were incubated for 30 min at 37°C with three different venom: IgY
216 ratios (1:16, 1:40, 1:100). Thereafter the mixture was intravenously injected into groups of 4 mice
217 per each mixture. Mice receiving only IgY antivenom or only venom diluted in saline solution were
218 also included as controls. The median effective dose (ED_{50}) was calculated considering the number
219 of dead mice within 96 h after the intravenous injection of the venom/antivenom mixture; results
220 were analyzed by means of the method of Spearman–Karber (Finney, 1971). The ED_{50} is expressed
221 in three different ways: µL of antivenom required to neutralize the challenge dose of venom that
222 was administered; µg of venom needed to neutralize 1 ml of antivenom (µg/mL); µg of venom
223 needed to neutralize 1 mg of antivenom (µg/mg). The specific activity of IgY was calculated as the
224 total protein concentration of IgY antivenoms needed to normalize the ED_{50} (da Silva and
225 Tambourgi, 2011).

226

227 **2.9 Statistical analysis**

228 Statistical analysis of data was performed with Minitab 18.1 (Minitab Inc.). Kolmogorov-
229 Smirnov test was used to verify the normality of the data. Kruskal Wallis test was performed for
230 Indirect ELISA and repeated measures one-way ANOVA followed by Tukey pairwise comparison
231 test was performed for chaotropic ELISA and protein concentration analysis. Statistical significance
232 was set at $P < 0.05$. For indirect, chaotropic ELISA and protein concentration data was presented as
233 mean ± SD of three independent experiments. Neutralization of hemorrhagic and necrotic activities

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234 was analyzed by non-linear regression. A polynomial curve was used to fit values in Bradford assay.
235 Data was plotted with GraphPad Prism 6.0 (GraphPad, USA).

236

237 **3. Results**

238

239 **3.1. Immunoreactivity of IgY anti- *B. alternatus***

240 Specific antibodies in serum were detected after the second immunization. Thereafter, from
241 the 3rd immunization onwards the level of antibodies reached a plateau that was maintained until the
242 end of the observation period (Figure 1). ELISA S/P ratio values of the IgY extracts against *B.*
243 *alternatus* venom ranged between 0.92 and 1.37 and no statistically significant difference was
244 observed between values of extracts produced at the end of the first cycle (3rd injection) and the
245 second cycle (4th and 8th injections) (Table 2). According to several authors (de Oliveira *et al*., 2011;
246 de Roodt *et al*., 1998; Lanari *et al*., 2010; Queiroz *et al*., 2008; Sousa *et al*., 2013) the
247 electrophoretic pattern of the venom registered was typical of *B. alternatus*. Analysis by Western
248 blot indicates that IgY extracts against *B. alternatus* recognized three protein complexes of high (>
249 40 kDa), medium (20-40 kDa) and low (< 20 kDa) molecular weights. These bands were not
250 recognized by pre-immune IgY extracts (data not shown). Bands of ~ 50 and 25 kDa were strongly
251 recognized than the other lower molecular weight bands (Figure 2).

252

253 **3.2. Purity of IgY extracts anti-*B. alternatus* venom**

254 Batches of IgY extracts were produced with yolks collected after the 3rd, 4th and 8th
255 immunizations from each hen. Protein pattern analyzed by SDS-PAGE under non-reducing
256 conditions showed intact IgY (~ 180 kDa) with good purity levels (Figure 3). Only traces of a
257 protein complex of ~ 35 kDa was found (< 10 %). On the other hand, electrophoretic patterns under
258 reducing conditions showed two protein complexes with a molecular weight of ~ 70 and 25 kDa
259 corresponding to the heavy and light IgY chains, respectively (Schade *et al*., 2005). Purity levels

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260 ranged from 82.9 to 88.2% and two bands of ~ 50 and 40 kDa were observed. Concentration of
261 sulphate contaminants was lower than 0.08% p/v. Total protein concentration of IgY extracts ranged
262 between 25.93 and 27.49 mg/mL after the 3rd injection, between 22.89 and 30.13 mg/mL after the
263 4th and between 19.40 to 33.49 after the latter.

264

265 **3.3. Avidity of IgY extracts anti- *B. alternatus***

266 The strength of the interaction between IgY and the venom was measured and significant
267 differences ($P < 0.05$) in the avidity indexes of the extracts obtained were observed after subsequent
268 immunizations (Table 2). After the last immunization avidity indexes of the extracts obtained from
269 each hen ranged from 84.10 to 93.01 %.

270

271 **3.4. Neutralization of venom**

272 No abnormal clinical signs or mortality were observed after the treatments in the control
273 group of mice that were injected with IgY antivenoms only. ED_{50} values of the IgY antivenoms
274 produced after the 4th and 8th immunization are shown in Table 3. After subsequent immunizations
275 lower volumes of antivenom were required to neutralize the lethal effect of the venom. Overall, IgY
276 antibodies neutralized from 12.10 to 35.65 µg of venom per mg of antivenom. In addition, highest
277 specific activity values were obtained after 8 injection doses, ranging from 0.28 to 0.42. The
278 neutralization of the hemorrhagic activity of the venom required 4.19 mg of IgY; in contrast, no
279 neutralization of the necrotic activity was observed.

280

281 **4. Discussion**

282

283 Treatment of snake envenoming by hyperimmune equine sera was established more than a
284 century ago (Gutiérrez *et al*., 2011; Marchand *et al*., 2013) and methods of production of
285 antivenoms have been considerably improved (Chippaux, 2013). So far, antivenoms are the only

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286 available therapeutic tool to treat envenoming by venomous animals (snakes, spiders, scorpions) (de
287 Roodt *et al*., 2004; Lachmann, 2012). Relative high costs of production make antivenoms
288 unaffordable for developing countries (Scheske *et al*., 2015) and therefore efforts to innovate and
289 simplify its production are encouraged worldwide (Gutiérrez *et al*., 2017a). In this context, we
290 produced and characterized experimental IgY-based antivenoms against *B. alternatus* venom.

291 IgY antibodies (~ 180 kDa) were the main components in the extracts analyzed under non-
292 reducing conditions, but also minor protein contaminants of around 35 kDa (< 10 %) were
293 observed. This contaminant traces may correspond to Gal d 6 (Amo *et al*., 2010; De Silva *et al*.,
294 2016). Nevertheless we did not observe the presence of the main allergen in the egg, Gal d 5
295 (Chalamaiah et al., 2017; Schade and Chacana, 2006) or ovalbumin (Gal d 2) (Campos *et al*., 2003;
296 Réhault-Godbert *et al*., 2013). On the other hand, under reducing conditions a contaminant of ~ 50
297 kDa was observed which may correspond to apolipoprotein B (Egger *et al*., 2011). Level of
298 purification as well as total protein content in the extracts is crucial to obtain efficient and safe
299 antivenoms. Contamination of undesired proteins in the IgY antivenoms can be reduced during the
300 scaling up of the purification steps. For example, use of caprylic acid combined with ammonium
301 sulphate is able to eliminate several contaminant proteins (Araújo *et al*., 2010; Mendoza *et al*.,
302 2012).

303 The production of antivenoms in horses against *B. alternatus* may take between 9 and 19
304 immunizations using high venom quantities (26.5-51 mg) (Araceli and Cheroni, 1994; de Roodt *et*
305 *al*., 2010). In our study significant levels of specific IgY in serum were detected by ELISA after the
306 second immunization; this early detection of antibodies in chickens was also observed by other
307 authors (Almeida *et al*., 1998; Moussa *et al*., 2012). After the 3rd immunization, levels of specific
308 IgY reached a maximum that was maintained throughout the observation period while, as expected,
309 avidity indexes of the extracts increased after successive immunizations (da Rocha *et al*., 2017; de
310 Andrade *et al*., 2013; Sampaio *et al*., 2014; Schade *et al*., 2005; Walczak *et al*., 2015). Anyhow,
311 despite ELISA may be useful to characterize the immunoreactivity of IgY, this assay does not infer

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312 the real potency of antivenoms (World Health Organization, 2017). Nevertheless, ELISA tests may
313 be helpful to evaluate immunization schemes by following up seroconversion as well as maturation
314 of the chicken humoral response. The diversity in the composition of *B. alternatus* venom is also
315 reflected in its immunogenic properties (Sousa *et al*., 2013). Our analysis showed that the IgY
316 extracts were able to recognize most of the main components of the venom that have been described
317 in the literature (Lanari *et al*., 2010). A band of ~ 50 kDa was strongly detected; this band may
318 correspond to class III svMPs, the most predominant toxin (~ 40 %) in *B. alternatus* (de Roodt *et*
319 *al*., 1998; Sousa *et al*., 2013). Moreover, IgY was also capable of detecting ~ 25 kDa bands
320 indicating that IgY was able to recognize svSPs and class I svMPs (Queiroz *et al*., 2008; Sousa *et*
321 *al*., 2013). Furthermore, IgY extract weakly recognized bands of low molecular weight (less than 15
322 kDa), which may include proteins such as phospholipase A_2 (PLA_2). This pattern of recognition was
323 similar to commercial or experimental equine-derived antivenoms (de Roodt *et al*., 1998; Sousa *et*
324 *al*., 2013). The relatively high amount of venom components, such as svMPs, may explain its high
325 antigenicity not only in birds but also in mammals (León *et al*., 2011; Öhler *et al*., 2010). In general,
326 toxin components of low molecular mass are less immunogenic as compared to the ones of high
327 molecular mass (Laustsen *et al*., 2017a). In this way, enhancing the humoral response is possible by
328 increasing the injection dosage or coupling the peptides with carrier proteins of high molecular
329 mass. For example, PLA_2s and three-finger toxins (3FTxs) have been reported to be capable of
330 eliciting an antibody response in chickens. da Rocha *et al*. (2017) produced IgY-based antivenoms
331 with high neutralizing activity as well as with a strong recognition of protein complexes with low
332 molecular mass that may include PLA_2 as analyzed by western-blot. On the other hand, Lee *et al*.
333 (2016a) obtained IgY antibodies capable of recognizing proteins of ~ 10 kDa showing that IgY is
334 able to detect 3FTxs. Also usage of venom-independent strategies such as synthetic peptide
335 epitopes, recombinant toxins or toxoids and DNA strings may allow the obtainment of therapeutic
336 antibodies with high neutralizing activity as demonstrated using mammal models (Bermúdez-
337 Méndez *et al*., 2018). These innovations may simplify the processes associated with the obtainment

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338 of the venoms by reducing (or even avoiding) the need of animals to collect them. In addition, they
339 could be combined with traditional immunization procedures due its compatibility with current
340 antivenoms manufacturing. In chickens, the use of innovative immunogens such as synthetic
341 peptides (Egea *et al*., 2018; Guevarra *et al*., 2012; Moreno *et al*., 2016), recombinant toxins (Hirai
342 *et al*., 2010; Mudili *et al*., 2015; Parma *et al*., 2012; J. You *et al*., 2014; Z. You *et al*., 2014) or DNA
343 strings (Kazimierczuk *et al*., 2005; Witkowski *et al*., 2009) have demonstrated the production of
344 specific antibodies with high titers, so they also should be explored to improve the production of
345 IgY-based antivenoms.

346 In this work, the performance of IgY antivenoms was evaluated using 3 LD_{50} as challenge
347 dose, according to the WHO guidelines (World Health Organization, 2017). IgY antibodies were
348 capable of neutralizing *B. alternatus* venom in mice. As it occurs in horses, not all hens are
349 expected to respond to the venom in the same way after the immunization (Angulo *et al*., 1997;
350 Gutiérrez *et al*., 1988) and thus, herein we analyzed the specific potency of these antivenoms per
351 each hen to assess individual variation in immune response. After 8 immunizations, 1 mg of
352 antivenom neutralized between 19.66 and 35.65 µg of venom among the hens studied. de Andrade
353 *et al*. (2013) produced a similar IgY antivenom against *B. alternatus* after 11 immunizations and
354 found a potency of 20.47 µg/mg. The immunization scheme used by the authors only considered
355 fixed doses of the venom (125 µg), and the antivenoms were produced by pooling eggs from
356 different hens, so no individual variability was analyzed. Also, levels of production may depend on
357 the genetic line of the laying hens: while de Andrade *et al*. (2013) produced the antivenom by
358 immunizing white leghorn hens; in our work brown laying hens were used. On the other hand, de
359 Roodt *et al*. (2010) immunized rabbits with *B. alternatus* and found that the potency achieved was
360 106.59 µg/mg. Furthermore, Segura *et al*. (2010) observed that the potency of commercial
361 bothropic antivenom was 72.01 µg/mg. In both cases, potency was from 3 to 8 times higher than the
362 antivenoms we have obtained after 8 immunizations. Nonetheless, potency of the IgY-based
363 antivenom increased throughout the immunizations scheme of the hens so it may be expected that

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364 with more boosters the potency could be improved and reach the values obtained using mammals.
365 Many variables may impact on the quality of the final antivenom. For example, amount and nature
366 of the antigen used, selection of the genetic line and the best responders among all immunized hens
367 may lead to improve the production.

368 Administration of antivenoms based on heterologous antibodies may induce side effects
369 such as hypersensitivity reactions and serum sickness, as has been observed when equine-based
370 antivenoms are used (Gutiérrez *et al.*, 2007). Parenteral administration of IgY may elicit undesirable
371 reactions and thus its immunogenicity should be also considered. In fact, Navarro *et al.* (2016)
372 found that rabbits developed higher levels of antibodies against IgY than against equine-IgG after
373 intravenous administration of the antivenoms. Comparative results of pharmacokinetic studies
374 conclude that IgY is more antigenic than equine-IgG (Díaz et al., 2014). Nevertheless, most
375 frequent early adverse reactions are induced by antivenoms with high protein concentrations and
376 immune complex aggregates (Gutiérrez *et al.*, 2011; Laustsen *et al.*, 2018b; Otero *et al.*, 1999) and
377 these factors may activate the complement system in human patients, mostly due to the Fc portion
378 of the heterologous immunoglobulins (Herrera *et al.*, 2005). Interestingly, some studies
379 demonstrated that IgY antibodies do not induce the mammal complement cascade neither *in vivo*
380 nor *in vitro* (Carlander and Larsson, 2001; Sesarman *et al.*, 2008). Anyhow, since human normal
381 plasma usually has higher titres of IgG against IgY than against equine-IgG, it is likely that more
382 frequent and severe late adverse reactions may be induced by IgY (Díaz *et al.*, 2014; León *et al.*,
383 2013; Sevcik *et al.*, 2008). However, no association between titers against heterologous antibodies
384 and antivenom safety was demonstrated (Herrera *et al.*, 2005; León *et al.*, 2008). On the other hand,
385 the use of Fab fragments of IgY (Sifi *et al.*, 2018) instead of the whole molecule may improve the
386 safety of IgY-antivenoms since heterologous Fab and $F(ab)_2$ molecules do not seem to elicit
387 anaphylactic reactions (Vázquez *et al.*, 2010).

388 The choice of any novel alternative approach should be selected by comparing the reduction
389 of undesirable effects due to the immunogenicity of either IgG or IgY. For instance, it was

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390 demonstrated that small molecules as Varespladib and Methyl-varespladib inhibit the activity of
391 snake PLA_2s (Lewin *et al*., 2016; Wang *et al*., 2018) or tetracycline inhibit the activity of
392 sphingomyelinase D, the main component of *Loxosceles* venom (Okamoto *et al*., 2017).
393 Nevertheless, even if the inhibition of small molecules has been effective against enzymatic toxins,
394 antibody-based antivenoms are more efficient to inhibit non-enzymatic toxins (Knudsen and
395 Laustsen, 2018). Usage of recombinant antibodies such as V_HH fragments (Alvarenga *et al*., 2014),
396 single-chain variable fragments (Roncolato *et al*., 2013), or human IgG antibodies produced by
397 CHO cell cultivation (Laustsen *et al*., 2017b) has also been explored. Furthermore, Laustsen *et al*.
398 (2018c) demonstrated that oligoclonal human IgG mixtures neutralize dendrotoxin-mediated
399 neurotoxicity of black mamba whole venom when mice were challenged by the
400 intracerebroventricular route but not when they were challenged by the intravenous route. Thus, the
401 authors suggest that human monoclonal IgGs cocktails should be carefully selected in order to
402 obtain an effective antivenom. Likewise, another study on recombinant antivenoms has
403 demonstrated the ability of a single human scFv to cross-neutralize the venom of five Mexican
404 scorpions (Riaño-Umbarila *et al*., 2019). Compared with heterologous polyclonal antibodies, such
405 as IgG or IgY-based antivenoms, the most significant advantage of human IgG-based recombinant
406 antivenoms is the compatibility with the human immune system. Also, the possibility of only
407 including antibodies of therapeutic value which may help to minimize adverse reactions.

408 Production costs could be reduced by the application of these alternative technologies,
409 although some limitations have been addressed such as the high cost of producing recombinant
410 biologics (Saeed *et al*., 2017; Stanton, 2018). Anyhow, considering the requirements of antivenoms
411 in sub-Saharan Africa, Laustsen *et al*. (2017b) estimated that the cost of treatment using
412 recombinant antivenoms produced in CHO cells would be between USD 60 and 250. This figure is
413 at least 2.5 times less costly than the price of the current antivenoms used in the sub-Saharan
414 African market that have a wholesale price of USD 640 per treatment. However, in most Latin
415 American countries antivenoms are mainly produced by non-profitable public institutions. For

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416 example, economic evaluation of equine-based snake antivenom production at the public health
417 system of Uruguay revealed that the cost per treatment for *B. alternatus* snakebite envenomation
418 ranges between USD 27.6 and 55.2 (Morais and Massaldi, 2006). Usage of IgY may specifically
419 reduce costs associated with the main source of immunoglobulins without requiring any significant
420 deviations from the traditional industrial processes (Navarro *et al*., 2016), so it is expected that
421 application of IgY-technology in Latin America would lower the cost per treatment than equine
422 IgG-based antivenoms. The production of effective and safe antivenoms still remain a major
423 challenge, but a possibility may exist for antivenoms based on recombinant antibodies and small
424 molecules inhibitors. Anyhow innovation in antivenom manufacture should be encouraged due to
425 their improved efficacy and safety even if their implementation may be difficult in developing
426 countries due to the costs associated with the expenses and complexity in production processes.

427 In conclusion, we produced and characterized IgY antivenoms capable of neutralizing the
428 lethal activity of *B. alternatus* venom at a preclinical level. Since all IgY extracts studied herein
429 have total protein contents lower than the officially approved requirements for equine-derived
430 antivenoms (World Health Organization, 1981), further concentration of chicken antibodies may
431 lead to an increase of their neutralizing potencies. Considering that specific antibodies represent
432 between 1 and 10 % of the total IgY (Schade *et al*., 2005), efficacy of the antivenoms can also be
433 improved either by instauration of proper affinity purification methods based on the whole venom
434 or usage of its main components as antigens during the immunization process. Although extra
435 purification steps may represent additional costs during the manufacture process, any undesirable
436 effects could be circumvented by reducing the amount of antibodies needed to neutralize a certain
437 amount of the venom. Thus, IgY-technology may allow the production of effective and affordable
438 antivenoms fulfilling the urgent needs of many countries where conventional manufacture is unable
439 to provide enough availability of antivenoms.

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441 **Competing Interests:**

442 The authors declare no conflicts of interest.

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1 **Tables**

2

3 **Table 1**

4

Number of immunizations	Days	Immunization dose (μg)	Adjuvant	Immunization Route
1	0	400	FCA	s.c. + i.m.
2	15	800	FIA	i.m.
3	30	1200	FIA	i.m.
	Rest period			
4	137	150	FIA	i.m.
5	151	300	FIA	i.m.
6	165	300	FIA	i.m.
7	179	300	FIA	i.m.
8	193	900	FIA	i.m.
	Total Venom	**4350**		

5

6 Table 1. Immunization scheme. s.c. = subcutaneous; i.m. = intramuscular

7

8 **Table 2**

9

Number of immunizations	Indirect ELISA (S/P ratio)	AI (%)	Protein concentration (mg/mL)
3	0.96 ± 0.04^{a}	69.42 ± 4.22^{a}	26.62 ± 0.80^{a}
4	1.14 ± 0.19^{a}	81.57 ± 5.99^{b}	26.38 ± 3.97^{a}
8	1.19 ± 0.19^{a}	89.48 ± 4.03^{c}	27.25 ± 5.89^{a}

10

11 Table 2. Immunoreactivity, avidity and protein concentration of IgY antivenoms. Values are shown
12 as mean ± SD. Different superscript letters in a column indicate significant differences between
13 numbers of immunizations ($P < 0.05$).

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17 **Table 3**

18

Number of immunizations	ED_{50}			Specific activity[b]
	(µL)[a]	(µg/mL)[b]	(µg/mg)[b]	
4	205.15 ± 85.14	460 [277 - 692]	19.21 [12.10 – 30.06]	0.19 [0.14 - 0.36]
8	116.30 ± 12.55	691.8 [691.8 - 870]	23.15 [19.66 – 35.65]	0.30 [0.28 - 0.42]

19

20 Table 3. Neutralization of lethality of IgY antivenoms, expressed in different ways. [a] ED_{50} data
21 shown as mean ± SD; [b]ED_{50} and specific activity is shown as median with minimal and maximal
22 values.

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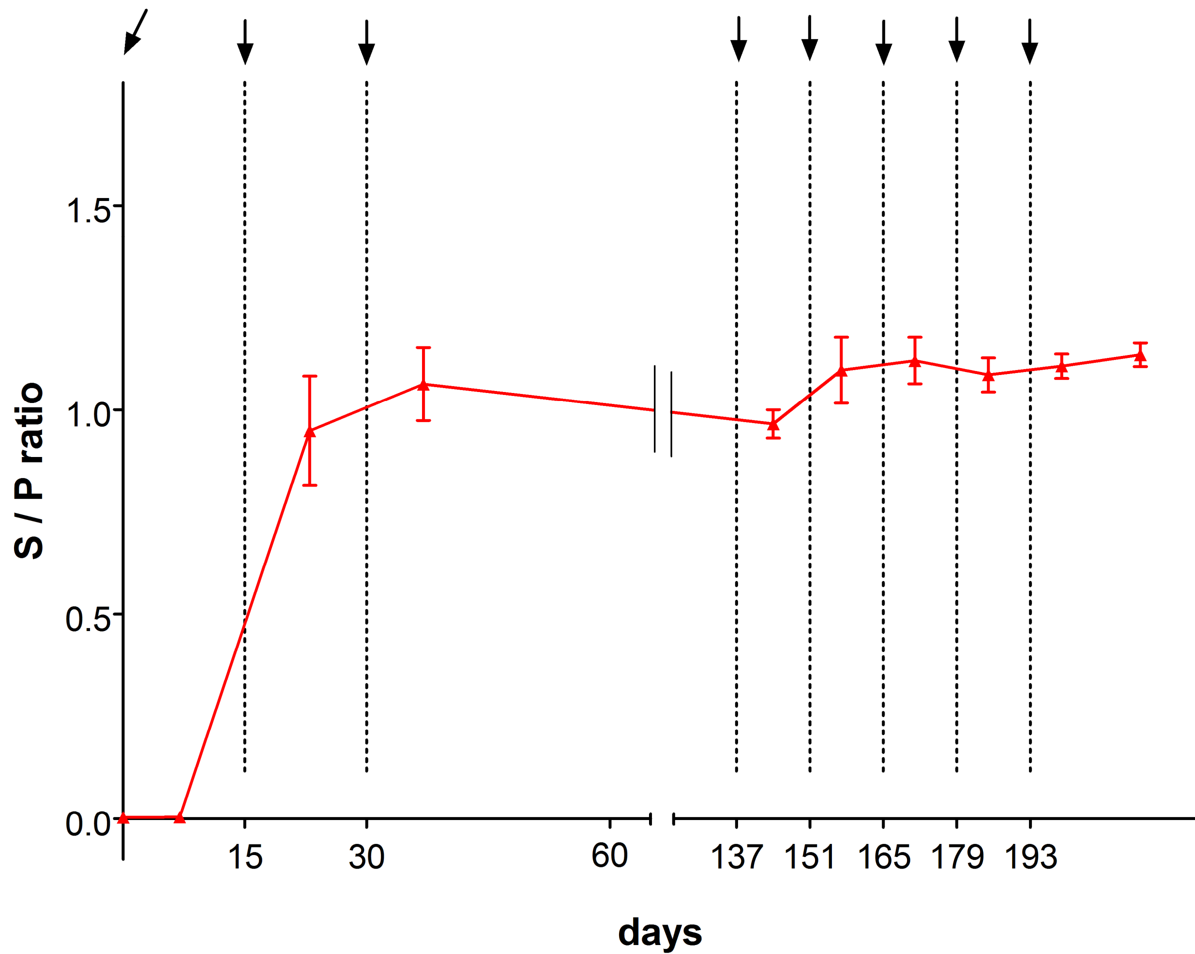
1 Figures

2

3 Figure 1

4



5

6 Figure 1. Relative level of IgY antibodies in serum of laying hens inoculated with *B. alternatus*

7 venom. Indirect ELISA (S/P ratio) is expressed as mean ± SD. Arrows indicate day of

8 immunizations.

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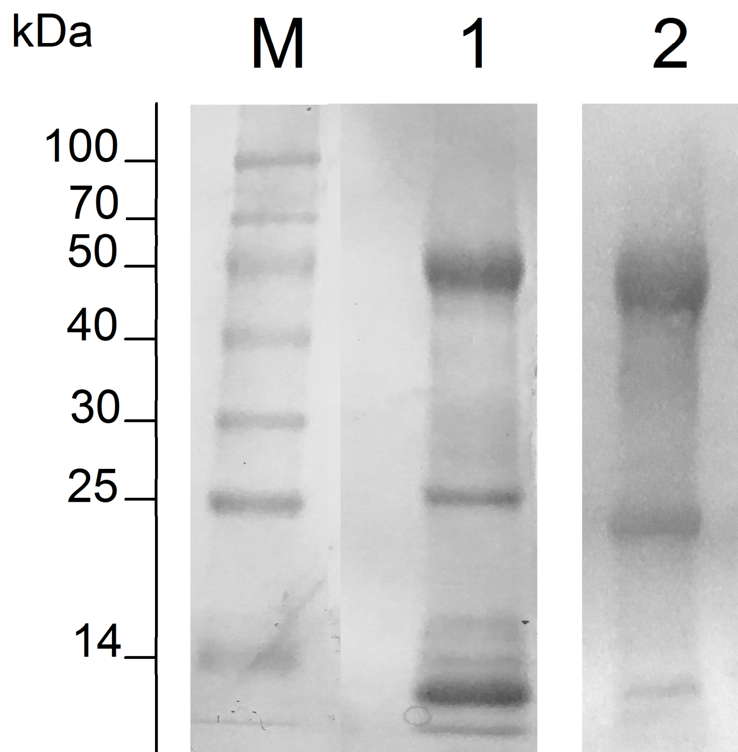
15 **Figure 2**



16

17 Figure 2. *B. alternatus* recognition by IgY antibodies. Lane M: protein ladder. Lane 1:
18 Electrophoretic profile (SDS-PAGE 15% under reducing conditions) of *B. alternatus* venom. Lane
19 2: *B. alternatus* compounds recognized by IgY extract obtained after the 8th immunization. This
20 figure is representative of several independent experiments.

21

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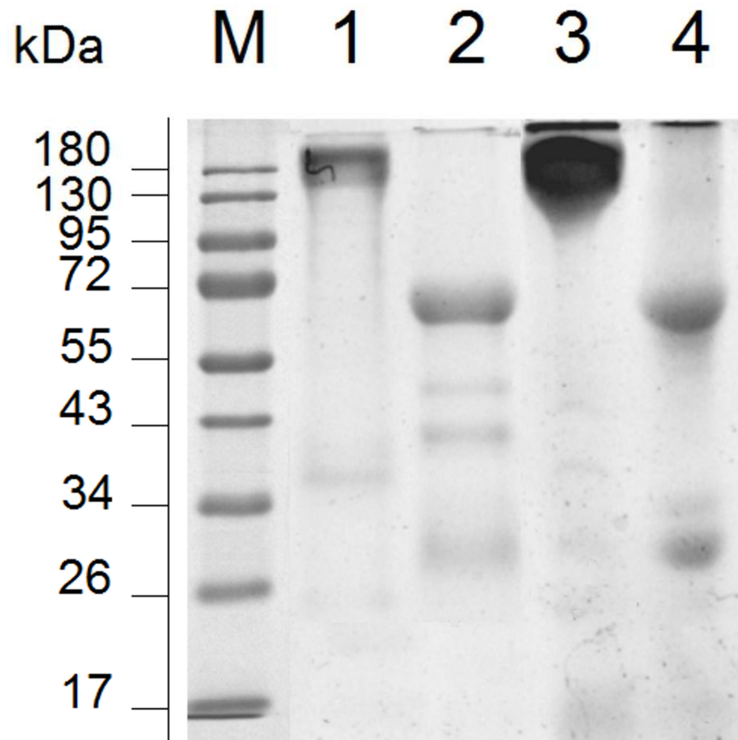
24 **Figure 3**



25

26 Figure 3. IgY extract analysis. Electrophoretic profile (SDS-PAGE 12%) of IgY extract. Lane M:
27 protein ladder. Lane 1 and 2: IgY extract obtained after the 8th immunization under non-reducing or
28 reducing conditions, respectively. Lane 3 and 4: IgY control (I4881; Sigma-Aldrich) under non-
29 reducing or reducing conditions, respectively. This figure is representative of several independent
30 experiments.

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Highlights

- We produced and characterized IgY antivenoms capable of neutralizing the lethal activity of *Bothrops alternatus* venom at a preclinical level.
- IgY against *B. alternatus* recognized protein complexes of the venom with high (>40 kDa), medium (20-40 kDa) and low (<20 kDa) molecular weights.
- After only 3 immunizations, hens produced IgY that neutralized *B. alternatus* venom.
- Avidity indexes of the IgY antivenoms increased after the successive immunizations.

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Ethical statement

This study meets the ARRIVE guidelines. The experiments were approved by the Institutional Animal Care and Use Committee (IACUC) from the CICVyA-INTA, Procedure #20/2012.

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