

Preclinical Evaluation of the Neutralising Efficacy of Three Monospecific Antivenoms Against the Venoms of Five African Echis Species, Including the Recently Partitioned *E. ocellatus* and *E. romani*

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1 **Full title:** Preclinical evaluation of the neutralising efficacy of three monospecific antivenoms against
2 the venoms of five African *Echis* species, including the recently partitioned *E. ocellatus* and *E. romani*

3
4 **Brief title:** Preclinical evaluation of paraspecific efficacy of three *Echis* monospecific antivenoms

5
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24
25 **Keywords:**

26 Snakebite envenoming; Antivenom; *Echis*; Venom; Preclinical

27
28 **Research in Context**

29 **Evidence before this study**

30 The recent partitioning of *Echis ocellatus* - the most medically important snake species in West Africa
31 – into *E. ocellatus sensu stricto* and *E. romani* raises questions as to whether existing antivenoms
32 raised against *E. ocellatus sensu lato* are effective against both species. We sought to determine the
33 preclinical efficacy of three monospecific antivenoms indicated for treatment of '*E. ocellatus*'

34 envenomings against *E. ocellatus s.str.* and *E. romani* venoms, and further compared the extent of
35 cross-species reactivity to three other medically important species of African *Echis*.

36 **Added value of this study**

37 Our study identifies variability in antivenom efficacy against the venoms of *E. ocellatus* and *E. romani*.
38 All three tested antivenoms recognised and bound to the diverse *Echis* venoms tested but
39 demonstrated strong differences in both *in vitro* and preclinical neutralising efficacy assays. The
40 antivenoms demonstrated some cross-reactivity beyond the venoms used in their manufacture,
41 however none of the monospecific *Echis* antivenoms were fully effective against *E. coloratus* from
42 North Africa at the doses tested.

43 **Implications of all the available evidence**

44 These findings provide preclinical evidence on the efficacy of three antivenoms in neutralising the
45 lethal effects of medically important *Echis* venoms from sub-Saharan Africa. Clinical evidence is
46 required to confirm these findings, but this work suggests that all three antivenoms may be effective
47 and could collectively meet the WHO recommendation to have three antivenoms available for
48 treatment of *Echis* envenomings in sub-Saharan Africa.

49 **Abstract**

50 **Background**

51 The genus *Echis* is of high medical importance across Africa. Recently the taxonomy of its most
52 medically important species, *Echis ocellatus*, underwent a revision, resulting in a splitting of the
53 species into *E. romani* and *E. ocellatus*, and leading to uncertainty of the efficacy of antivenoms
54 indicated for treatment of '*E. ocellatus*' envenomings against the two redefined species.

55 **Methods**

56 We compared the *in vitro* and murine preclinical venom-neutralising efficacy of three antivenoms
57 (EchiTabG, SAIMR Echis and Echiven) raised against *E. ocellatus sensu lato* against the venoms of *E.*
58 *romani* and *E. ocellatus*, and investigated cross-reactivity to *E. coloratus*, *E. leucogaster*, and *E.*
59 *pyramidum leakeyi*.

60 **Findings**

61 In preclinical assays of envenoming, all three antivenoms neutralised Nigerian *E. romani* venom,
62 though all three were less protective against Cameroonian *E. romani*. SAIMR Echis and Echiven
63 neutralised *E. ocellatus* venom whereas EchiTabG was less protective. SAIMR Echis and Echiven
64 showed strong cross-reactivity to *E. p. leakeyi* and *E. leucogaster*, whilst EchiTabG showed weaker
65 cross-reactivity. All three antivenoms exhibited poor neutralisation of *E. coloratus* venom.

66 **Interpretation**

67 This represents the first detailed analysis of differences between *E. ocellatus* and *E. romani* venom
68 bioactivities and the impact of antivenom on these two species. Our findings demonstrate that SAIMR
69 Echis and Echiven antivenoms are preclinically efficacious against the lethal effects of several species
70 of *Echis*. These products, in addition to EchiTabG, seem likely to meet the WHO recommendation of
71 three antivenoms required for treatment of *Echis* envenomings across sub-Saharan Africa, though
72 clinical evidence is required to confirm these findings.

73 **Funding**

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76 1. Introduction

77 Snakebite envenoming, a World Health Organization (WHO) recognised Neglected Tropical Disease,
78 is estimated to affect 2.7 million people each year, with global annual rates of up to 138,000 deaths
79 and over 400,000 people suffering life-altering morbidity [1]. Rural impoverished populations across
80 the tropics predominantly suffer the greatest burden from snakebite envenoming [2]. The WHO is
81 currently implementing a strategy to tackle the burden of snakebite envenoming, with the target of
82 reducing snakebite death and disability by 50% by 2030 [3,4]. To achieve this ambitious target, one of
83 the strategic objectives is to ensure provision of safe and effective antivenoms capable of treating
84 envenoming from the most medically important snakes globally. Amongst other approaches, the WHO
85 is currently performing risk-benefit analyses of antivenom products to recommend three fit-for-
86 purpose and quality-assured antivenoms per geographical region, initially beginning with sub-Saharan
87 Africa [3].

88
89 Snakes of the genus *Echis* (common name: saw-scaled or carpet vipers) are one of the most medically
90 important groups of snakes responsible for a large proportion of global snakebite burden [5], currently
91 consisting of 13 recognised species found throughout much of Africa and extending through the
92 Middle East to India and Sri Lanka [6]. Saw-scaled vipers are estimated to be responsible for two-thirds
93 of snakebite envenoming in West Africa [7], with *E. ocellatus* historically invoked as the species
94 responsible for most of the mortality and morbidity from snakebite in this region [8].
95 Pathophysiological consequences of envenoming by the *Echis* genus are characteristic of viperid
96 snakes, consisting predominately of haemotoxic effects defined by frequent bleeding disturbances,
97 venom-induced consumption coagulopathy (VICC) and local tissue necrosis [9,10].

98
99 In 2009, a molecular phylogenetic analysis of the genus *Echis*, including *E. ocellatus* from multiple
100 regions in West Africa, demonstrated the presence of three distinct phylogroups within the species
101 and, given the large degree of morphological variation within the species, led the authors to
102 hypothesise that “additional organismal lineages” may exist [6]. Based on this molecular phylogeny
103 and the analysis of pattern and scalation characters, *E. ocellatus* was partitioned into two species, *E.*
104 *ocellatus* and *E. romani*, in 2018 [11]. The distribution of *E. ocellatus* is now thought to extend from
105 eastern Guinea to north-western Nigeria, while *E. romani* is thought to be resident in northern and
106 north-eastern Nigeria east to at least southern Chad [6,11], with an apparently isolated population in
107 Sudan [12]. The partition of the historic *E. ocellatus* species into *E. ocellatus sensu stricto* and *E.*
108 *romani*, with their geographically distinct regions, has led to uncertainty about the efficacy and cross-
109 reactivity of current antivenoms against the venom of these two newly defined species. For clarity,

110 we will refer to *E. ocellatus* in its old, pre-partition sense, i.e., including *E. romani*, as *E. ocellatus sensu*
111 *lato*, whereas the post-partition interpretation of *E. ocellatus* will be referred to as *E. ocellatus sensu*
112 *stricto*.

113

114 The only effective therapy for treating snakebite envenoming is antivenom, a polyclonal antibody-
115 based serotherapy generated by immunising large animals (equines/ovines) with crude venom to
116 produce anti-toxin antibodies [13,14]. Antivenoms are commonly raised against multiple species of
117 venom to produce 'polyvalent' antivenoms, however for some of the most medically-important
118 species 'monospecific' or 'monovalent' antivenoms raised against a single species may be
119 manufactured. Venom toxin variation among snake species dictates that different antivenoms may be
120 required to effectively treat bites by different snake species [15–17]. Currently there are three
121 monospecific antivenoms designed for use against the *Echis* species present in sub-Saharan Africa;
122 EchiTABG, SAIMR Echis and Echiven – all of which were manufactured using *E. ocellatus sensu lato*
123 venom [18]. The effectiveness of antivenoms in neutralising saw-scaled viper envenoming in Nigeria
124 has been particularly well demonstrated, following the success of the EchiTab Study Group's
125 manufacture and randomised clinical trial of two antivenoms against *E. ocellatus sensu lato* in the
126 country [9,19]. As of 2020, the efficacy of nine different monovalent and polyvalent antivenoms raised
127 against, or with suggested efficacy via cross-reactivity against, *E. ocellatus sensu lato* had been
128 examined in 30 different preclinical studies [20], and the findings of this analysis demonstrated a wide
129 range of reported efficacy, both between the different antivenoms and sometimes for the same
130 antivenom against the same species [19,21]. Whilst EchiTABG and SAIMR Echis antivenoms have been
131 robustly independently examined for clinical efficacy against pre-taxonomic partition *E. ocellatus*
132 envenoming, they have not been directly compared against each other for clinical neutralising
133 efficacy. Echiven however is a recent addition to the sub-Saharan African market and to date has not
134 been tested in clinical trials and no publicly available preclinical data exist.

135

136 In addition to *E. ocellatus*, other species of *Echis* present Africa can cause serious envenoming, and
137 these include *E. coloratus* from north-east Africa, *E. leucogaster* from north and west Africa and *E.*
138 *pyramidum leakeyi* from east Africa, although the latter is not necessarily restricted to this species
139 alone, but this subspecies is the most commonly documented. These venoms are not usually
140 specifically indicated for neutralisation by current available *Echis* monospecific antivenoms, yet
141 paraspecific neutralisation has been demonstrated in a small number of preclinical and clinical studies.
142 However, these comprise several independent studies focusing on specific venoms or antivenoms,
143 thus confounding comparative interpretations [22–26].

144

145 The recent partition of *E. ocellatus* means that it is likely that several existing antivenoms indicated
146 for *E. ocellatus* envenoming may actually have been manufactured using *E. romani* venom or using a
147 mixture of *E. romani* and *E. ocellatus* venom. Detailed analyses of the biological differences between
148 *E. ocellatus* and *E. romani* venom remain outstanding and therefore it remains to be demonstrated
149 whether existing antivenoms have different efficacies in neutralising the venom of each species.
150 Consequently, we sought to determine if the snakes and venoms used to manufacture EchiTABG
151 antivenom, which were collected in northern Nigeria and maintained at the Liverpool School of
152 Tropical Medicine (LSTM) as part of the EchiTAb study group, were indeed *E. ocellatus*, or *E. romani*,
153 or a mixture of both species. We then investigated and directly compared the paraspecific neutralising
154 efficacy of three *Echis* monospecific antivenoms (EchiTABG, SAIMR Echis and Echiven) in robust *in vitro*
155 and *in vivo* preclinical assays against a broad range of African *Echis* species (*E. ocellatus*, *E. romani*, *E.*
156 *leucogaster*, *E. p. leakeyi* and *E. coloratus*).

157

158 **2. Methods**

159 2.1 Venoms

160 Venoms of *E. coloratus* (Egypt), *E. p. leakeyi* (Kenya), *E. romani* (Nigeria) and *E. leucogaster* (Mali) were
161 obtained from venom stocks from snakes either housed or previously housed in the herpetarium at
162 LSTM. Venoms of *E. romani* from Cameroon (sold as *E. ocellatus*, hereafter referred to as *E. romani*
163 [Cameroon]) and *E. ocellatus* from Ghana were purchased from Latoxan, France (Product ID L1114 for
164 both). Venoms were stored as lyophilised powders at 4 °C until reconstitution, and were reconstituted
165 in PBS pH 7.4 (Gibco, UK #10010) to 10 mg/mL stocks and aliquoted for storage at -80 °C.

166

167 2.2 Total DNA extraction and Sanger sequencing

168 Total DNA was isolated from 40 individual snakes originating from the Kaltungo (Gombe) region of
169 north-eastern Nigeria that were collected between April 2008 and September 2014 and housed in the
170 LSTM herpetarium as part of the EchiTAb study group collection. The DNA was sourced from individual
171 skin sheds for those snakes currently held in captivity at the time of this study, or historical lyophilised
172 venom samples extracted from individual snakes previously held in the collection. Additionally, a shed
173 skin from one *Echis carinatus* (Pakistan) specimen and lyophilised venoms from *E. ocellatus* (Ghana)
174 and *E. romani* (Cameroon) pooled from individual specimens, purchased from Latoxan (France) were
175 examined. Venoms were resuspended in PBS pH 7.4 (Gibco, UK #10010) prior to DNA extraction. Total
176 DNA was isolated using a DNeasy Blood & Tissue Kit (Qiagen, UK, #69506) following the manufacturers
177 recommended “tissue extraction protocol” for shed snake skin (using ~2.5 mg of skin) and the “blood

178 extraction protocol” for resuspended venom (starting with 100 μ L of 10 mg/mL w/v venom). PCR of
179 *NADH4* and *CYTB* was performed using primer pairs GludgMod2/EchR for *CYTB* and NADH4/EchR for
180 *NADH4* as described in [7], at a final concentration of 0.5 μ M per primer with Phusion Green Hot Start
181 II High-Fidelity PCR Master Mix (ThermoFisher UK, #F566S). Approximately 1 ng of template DNA was
182 used per reaction. PCR was performed on a 5PRIMEG/02 or TC-512 thermocycler (both Techne [Cole-
183 Parmer], UK), with the following cycle conditions: initial denaturation (98 $^{\circ}$ C, 30 s), followed by 30
184 cycles of denaturation (98 $^{\circ}$ C, 10 s), annealing (50 $^{\circ}$ C, 10 s) and extension (72 $^{\circ}$ C, 15 seconds), followed
185 by a final extension step (72 $^{\circ}$ C, 300 s). Total DNA extracted from venom solutions was typically very
186 low yield (<1 ng/ μ L), resulting in poor PCR amplification of both markers. To obtain suitable yields of
187 DNA for sequencing, reactions were purified using a QIAquick PCR Purification Kit (Qiagen, UK #28104)
188 and eluted material used as a template in a second round of PCR using the same conditions as the first
189 round. Resulting amplified DNA was subsequently purified using a QIAquick PCR Purification Kit
190 (Qiagen, UK #28104) and quality checked by 1% agarose (Severn Biotech, UK #30-10-10) gel
191 electrophoresis (180 V, 30 minutes) in 1 X Tris-acetate EDTA buffer (Severn Biotech, UK #20-6001-10,
192 final composition 40 mM Tris-acetate, 10 mM EDTA, pH 8.0). Amplicons were Sanger sequenced by
193 Source Bioscience (Cambridge, UK) using respective amplicon primer sets, above.

194

195 2.3 Phylogenetic analysis

196 Resulting sequences were quality checked and aligned using MEGA 11 [27] (RRID:SCR023017). To
197 provide a phylogenetic reference framework, we included in the alignment all sequences of the *E.*
198 *ocellatus* group (*E. ocellatus*, *E. romani*, *E. jogeri*), sequences of an *E. carinatus* from the United Arab
199 Emirates, and, as outgroup, sequences of a specimen of *Cerastes cerastes*, which were all sequenced
200 and published as part of a previous phylogenetic study of *Echis* [30]. We implemented the Model
201 function in MEGA 11, using the Bayesian Information Criterion (BIC) to identify the best substitution
202 model for the unpartitioned data prior to Maximum Likelihood (ML) phylogenetic analysis with 100
203 bootstrap replicates.

204

205 2.4 Antivenoms and control immunoglobulins

206 Antivenoms used were; (i) EchiTABG (whole ovine IgG manufactured by MicroPharm UK Ltd) raised
207 against saw-scaled viper venom of Nigerian origin classified at the time as *E. ocellatus* (venom
208 provided by LSTM), (ii) snake venom antiserum (*Echis* “Echiven” (equine F[ab]₂ manufactured by VINS
209 Bioproducts Ltd, India) raised against the venom of saw-scaled vipers from Cameroon, Ghana and Mali
210 (provided by Latoxan, France, all listed as *E. ocellatus*) and (iii) “SAIMR *Echis carinatus*” antivenom
211 (equine F[ab]₂ manufactured by South African Vaccine Producers PTY, South Africa) raised against *E.*

212 *ocellatus* of unknown geographical origin. Note that all African saw-scaled vipers were formerly
 213 included in *E. carinatus* prior to multiple taxonomic revisions – *E. carinatus* now refers solely to the
 214 species *E. carinatus*, found from the United Arab Emirates, Iraq and Turkmenistan south-east to India
 215 and Sri Lanka, while African *Echis* were split into several new species [6] and *E. ocellatus* has since
 216 been split into *E. ocellatus* and *E. romani*. Antivenoms EchiTabG and SAIMR Echis were donated to
 217 Liverpool School of Tropical Medicine by UK health authorities post expiry, whilst a sample of Echiven
 218 was kindly donated by VINS Bioproducts Ltd. The lyophilised Echiven was resuspended in 10 mL sterile
 219 water (provided by the manufacturer) prior to use. Control equine F(ab)₂ for incorporation as a
 220 control in the *in vitro* experiments described below was produced from equine IgG (BioRad #PEP001)
 221 using the Pierce F(ab)₂ Preparation Kit (Pierce, ThermoScientific #44988) according to manufacturer's
 222 protocols.

223
 224 Antivenom protein concentration was determined using a Pierce BCA Protein Assay kit (ThermoFisher,
 225 #23225), using known concentrations of purified ovine and equine IgG (BioRad #PSP01 and #PEP001,
 226 respectively) to produce ovine and equine IgG standard curves. To determine the protein
 227 concentration of EchiTabG, data were interpolated from the ovine IgG standard curve, and to
 228 determine the protein concentration of SAIMR Echis and Echiven, data were interpolated from the
 229 equine IgG standard curve. The BCA assay was performed according to the manufacturer's protocols
 230 using the microplate assay with an incubation at 37 °C for 30 minutes, with each test condition tested
 231 in duplicate. All three antivenoms were diluted 1 in 100 and 1 in 200 in PBS (Gibco, #20012, pH 7.4)
 232 before assaying, to dilute the antivenoms to fall within the standard curve and working range of the
 233 BCA assay, and the mean protein concentration estimate from the two dilutions was determined in
 234 Excel (Microsoft, RRID:SCR_016137) (data available in Supp. File 1). An overview of antivenoms used
 235 in this study is displayed in Table 1.

236
 237 **Table 1. An overview of the antivenoms used in this study.** The table shows manufacturer
 238 information, batch/lot numbers and expiry dates, composition of antivenom immunoglobulins and
 239 protein concentration and standard deviation as determined by BCA assay, and the venoms against
 240 which the manufacturers claim the antivenoms are effective. The asterisk denotes what is indicated
 241 on the inserts of the products and is assumed *E. ocellatus sensu lato*.

Antivenom tested	Manufacturer	Batch/Lot & Expiry date	Preparation and protein concentration of antivenom	Stated efficacy according to product insert
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EchiTabG	MicroPharm Ltd (UK)	- EOG 001440 - January 2017	- Ovine - Liquid - Intact immunoglobulins - 33.8 ± 7.5 mg/mL	West African saw-scaled of carpet viper, <i>E. ocellatus</i> *
SAIMR "Echis carinatus"	South African Vaccine Producers (SAVP) PTY, South Africa	- BC 00147 - January 2016	- Equine - Liquid - F(ab') ₂ fragment of immunoglobulins - 84.8 ± 20.4mg/mL	Saw-scaled viper <i>E. carinatus</i> / <i>ocellatus</i> * and paraspecific against <i>E. coloratus</i> and two species of <i>Cerastes</i>
Snake Venom Antiserum (Echis) ("Echiven")	VINS BioProducts Ltd, India	- 38AS21001 - October 2025	- Equine - Lyophilised powder - F(ab') ₂ fragment of immunoglobulins - 47.6 ± 5.0 mg/mL	<i>E. ocellatus</i> *

242

243 2.5 End-point ELISA

244 Venoms from six *Echis* species (*E. coloratus*, *E. leucogaster*, *E. ocellatus* [Ghana], *E. romani* [Nigeria],
245 *E. romani* [Cameroon], *E. p. leakeyi*) were coated at a concentration of 100 ng per well onto Nunc
246 MaxiSorp ELISA plates (ThermoFisher) in 50 mM carbonate-bicarbonate coating buffer pH 9.5 (Sigma
247 #C3041) and allowed to bind for one hour at 37 °C. Plates were washed six times with Tris-buffered
248 saline with 0.1% Tween20 (TBS-T), and then blocked with 5% milk in TBS-T for two hours at room
249 temperature. Plates were washed three times in TBST before each antivenom (neat, non-normalised)
250 was diluted 1 in 500 in blocking solution, added to the plate and five-fold serial diluted six times before
251 being incubated overnight at 4 °C. The following day, plates were washed six times in TBS-T and anti-
252 horse or anti-sheep IgG secondary antibodies conjugated to horseradish peroxidase (Sigma #A6917
253 and #A3415, respectively) were added at 1 in 1000 dilution in PBS for two hours at room temperature.
254 Plates were washed six times with TBS-T and developed with ABTS substrate (0.1 mg/mL 2,2'-azino-
255 bis[3-ethylbenzthiazoline-6-sulfonic acid] diammonium salt [Sigma #A9941] in 0.05 M citrate buffer
256 pH 5.0 with 0.0075% hydrogen peroxide) for 15 minutes at room temperature. The optical density was
257 immediately read at a wavelength of 405 nm (OD₄₀₅) on a LT-4500 plate reader (Labtech). All
258 measurements were performed in duplicate, and control wells consisting of venom-naïve sheep IgG
259 or horse F(ab')₂ (diluted 1 in 25 or 1 in 5 in PBS respectively to match average protein concentration
260 of the antivenoms at 1 in 500 dilution, then serial diluted as per antivenom), as well as secondary
261 antibody only, were also included. Data are available in Supp. File 2.

262

263 2.6 Phospholipase A₂ assay

264 Neutralisation of venom phospholipase A₂ (PLA₂) activity was measured using an EnzCheck
265 Phospholipase A₂ assay kit (Invitrogen, UK #E10217), as previously described [28]. All test conditions
266 were assayed in triplicate for all plates, and plates were measured on a CLARIOstar (BMG Biotech) at
267 excitation 485-15 nm and emission 520-10 nm. For all data analyses, buffer only well values were
268 subtracted from all other values as per manufacturer instructions. A bee venom PLA₂ standard curve
269 (provided in the assay kit) was ran in each assay plate in accordance with manufacturer instructions
270 to determine the specific PLA₂ enzymatic activity, and the specific activity of test conditions were
271 determined by interpolation from the equation of the bee venom PLA₂ standard curve (plotted in
272 Prism 9, GraphPad, RRID:SCR_002798). Optimisation of the amount of venom to be used for each
273 species was first performed to identify the amount of venom that falls within the linear range of
274 enzymatic activity measurements. The relative fluorescence units (RFU) were plotted against the
275 amount of venom per well, and the graphs were manually assessed to identify venom amounts in the
276 linear range of the assay. From these results (shown in Supp. Fig. S1 and Supp. File 3), the optimal
277 venom amounts were determined as 1 µg for *E. romani* (Cameroon), *E. ocellatus* (Ghana) and *E.*
278 *romani* (Nigeria), 0.5 µg for *E. coloratus*, and 0.25 µg for *E. leucogaster* and *E. p. leakeyi*. RFU was
279 converted to specific PLA₂ enzymatic activity (U/mL/µg) in Excel (Microsoft, RRID:SCR_016137) using
280 the equation derived from the bee venom PLA₂ standard curve to compare the PLA₂ activity of the
281 venoms.

282
283 Antivenoms were serial diluted two-fold (in PBS containing the pre-defined amount of venom to 12.5
284 µL volume per well) in a clear, polystyrene 384-well plate (Greiner BioOne #781101). Final volumes of
285 antivenom in respective wells were 0.10 µL to 6.25 µL. Plates were incubated at 37 °C for 30 minutes
286 then cooled to room temperature, following which 12.5 µL PLA₂ substrate (reconstituted as per
287 manufacturer instructions) was added to each well. Plates were incubated in the dark at room
288 temperature for 10 minutes and then read in a CLARIOstar plate reader (BMG Labtech). RFU
289 measurements were converted to PLA₂ activity using the equation of the standard curve, and then
290 expressed as percentage of activity (where the venom only control was 100% activity) using Microsoft
291 Excel (Microsoft, RRID:SCR_016137). For statistical analyses the data were analysed using two-way
292 ANOVA (multiple comparisons) in Prism 9 (GraphPad, RRID:SCR_002798) to compare the antivenoms
293 at each dilution. For clarity, data shown are the highest four amounts of antivenom, and data for the
294 lower amounts of antivenom are in Supp. File 3.

295

296 2.7 Snake venom metalloproteinase assay

297 Snake venom metalloproteinase (SVMP) activity and neutralisation of the six *Echis* venoms was
298 measured using the previously described fluorogenic peptide assay [29]. Briefly, 1 μL of 500 $\text{ng}/\mu\text{L}$
299 venom or equal volume of PBS was added to each well in a clear, polystyrene 384-well plate (Greiner
300 Bio-One), followed by 10 μL of antivenom (at dilutions of 1 in 4, 1 in 8, 1 in 16 and 1 in 32 equating to
301 2.5, 1.25, 0.625 and 0.313 $\mu\text{L}/\text{well}$) or an equal volume of PBS. Venom only, antivenom only and PBS
302 only controls were included. The 6.2 mM SVMP substrate ES010 (BioTechne) was diluted in reaction
303 buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5) to a 7.86 μM substrate solution. The assay plate was
304 incubated at 37 $^{\circ}\text{C}$ for 25 minutes and then placed at room temperature for 5 minutes before the
305 addition of 90 μL SVMP substrate solution to each well (7 μM final well concentration in the final well
306 volume of 101 μL). The plate was immediately read at excitation 320-10 nm and emission 420-10 nm
307 with automatic gain for 75 minutes on a CLARIOstar plate reader (BMG Labtech). All conditions were
308 performed in replicates of four within the plate. For analysis, the RFU at 60 minutes was analysed.
309 SVMP activity was calculated for each venom, in which 'venom only' wells represent 100% activity and
310 the change in SVMP activity in the presence of the test antivenoms was calculated as a percentage of
311 the 'venom only' wells. Ordinary one-way ANOVA was performed in Prism 9 (GraphPad,
312 RRID:SCR_00279) and Tukey's multiple comparison post-hoc test was performed on pairwise
313 comparisons. Data are available in Supp. File 4.

314

315 2.8 Bovine plasma clotting assay

316 Plasma clotting activity of the six *Echis* venoms and neutralisation by antivenoms was measured using
317 a previously described bovine plasma clotting assay [30,31]. Briefly, 1 μL of 100 $\text{ng}/\mu\text{L}$ venom or equal
318 volume of PBS was added to each well in a clear, polystyrene 384-well plate (Greiner Bio-One),
319 followed by 10 μL of antivenom (at dilutions of 1 in 4, 1 in 8, 1 in 16 and 1 in 32 equating to 2.5, 1.25,
320 0.625 and 0.313 $\mu\text{L}/\text{well}$) or equal volume of PBS. Venom only, antivenom only and PBS only controls
321 were also included, and all conditions were performed in replicates of four within the plate. The assay
322 plate was incubated at 37 $^{\circ}\text{C}$ for 25 minutes then room temperature for 5 minutes, before 20 μL of 20
323 mM calcium chloride (Sigma, #C1016) followed by 20 μL of citrated bovine plasma (Biowest, VWR
324 #S0260) was added to each well. The optical density was immediately read at a wavelength of 595 nm
325 (OD_{595}) for 115 minutes on a CLARIOstar plate reader (BMG Labtech). For analysis, the cross-section
326 at which the 'normal plasma clotting' curve intersected the curves of the test conditions was manually
327 identified and the area under the curve at this time point for each condition was calculated
328 (normalised to venom and PBS only controls) before converting to percentage activity as described in
329 the SVMP assay in Section 2.7. Ordinary one-way ANOVA was performed in Prism 9 (GraphPad,

330 RRID:SCR_002798), and Tukey's multiple comparison post-hoc test was performed on pairwise
331 comparisons. Data are available in Supp. File 5.

332

333 2.9.1 Ethical approvals

334 Animal experiments were conducted under protocols approved by the Animal Welfare and Ethical
335 Review Boards of the Liverpool School of Tropical Medicine and the University of Liverpool, and under
336 project licence P24100D38 approved by the UK Home Office in accordance with the UK Animal
337 (Scientific Procedures) Act 1986.

338

339 2.9.2 Animal maintenance

340 CD1 mice (male, 18-20 g, Charles River UK) were grouped in cages of five upon arrival (forming the
341 experimental unit) and acclimated for one week before experimentation in specific pathogen-free
342 conditions. No further randomisation was conducted. Holding room conditions were 23°C with 45-
343 65% humidity and 12/12 hour light cycles (350 lux). Mice were housed in Techniplast GM500 cages
344 (floor area 501 cm²) containing 120 g Lignocell wood fibre bedding (JRS, Germany), Z-nest
345 biodegradable paper-based material for nesting and environmental enrichment (red house, clear
346 polycarbonate tunnel and loft). Mice had *ad lib* access to irradiated PicoLab food (Lab Diet, USA) and
347 reverse osmosis water in an automatic water system. Cages were changed fortnightly with fresh
348 material in the new cage. Cages were selected at random for experimental treatments, and all
349 experiments used mixed gender experimenters who were unblinded to the test articles.

350

351 2.9.3 ED₅₀ and comparative ED₁₀₀ experiments

352 The median effective dose (ED₅₀) assay was performed to determine the dose of antivenom (μL) that
353 prevented venom-induced lethality in 50% of animals injected with 5 x the median lethal dose (LD₅₀:
354 the venom challenge dose that causes lethality in 50% of animals) of venom. The dose of antivenom
355 in these assays which prevented lethality in 100% of animals injected with 5 x LD₅₀ of venom was
356 thereafter used as the ED₁₀₀. In all experiments doses of venom and antivenom were pre-mixed and
357 incubated at 37 °C for 30 minutes prior to intravenous injection via the tail vein. No inclusion or
358 exclusion criteria were set. Groups of five mice were used for LD₅₀ and ED₅₀ experiments as per WHO
359 assay guidelines [13] except in the case of missed or partial doses during injection, as indicated in
360 Supp. File 6 and Supp. File 7 – in these instances group size for the purposes of data analysis was four
361 mice (n=27 for LD₅₀ and 117 for ED₅₀ in total). Confounders were not controlled. All ED₁₀₀ experiments
362 used groups of five (n=60 in total). The LD₅₀ values were identified from the literature and are shown
363 in Table 2.

364

365 **Table 2. Median Lethal Dose (LD₅₀) and the subsequent 5 x LD₅₀ for murine lethality model.** Reported
366 LD₅₀ values for each of the six *Echis* venoms (with source of reported LD₅₀), and the 5 X LD₅₀ dose used

Venom	LD50 (µg/mouse)	5 X LD ₅₀ (µg/mouse)
<i>E. coloratus</i> (Egypt)	9.81 [22]	49.05
<i>E. leucogaster</i> (Mali)	24.90 [26]	124.50
<i>E. ocellatus</i> (Ghana)	18.20 [32]	91.00
<i>E. romani</i> (Cameroon)	33.10 [21]	165.50
<i>E. romani</i> (Nigeria)	17.85 [33]	89.25
<i>E. p. leakeyi</i> (Kenya)	13.55 [22]	67.75

367 for effective dose₅₀ (ED₅₀) and ED₁₀₀ experiments in this study.

368

369 Animals were continuously monitored throughout the six-hour experiment for symptoms of systemic
370 venom toxicity (starred coat, grimace, hunching, slumping, decreased movement, respiration
371 abnormalities, strength of grip and maintenance of righting reflex, body temperature) and reaching
372 humane endpoints (HEP) (seizure, nasal haemorrhage or loss of righting reflex). Upon reaching HEPs,
373 animals were euthanised using rising concentrations of carbon dioxide or cervical dislocation. Time to
374 HEP, number of deaths and number of survivors were recorded.

375

376 The protocols were prepared before the study with the research questions of i) determining ED₅₀ and
377 ED₁₀₀ of the three antivenoms against *E. romani* (Nigeria) venom and *E. p. leakeyi* (Kenya) venom and
378 (ii) assessing the survival rates of animals injected with a) *E. romani* (Cameroon) and *E. ocellatus*
379 (Ghana) when given the ED₁₀₀ of antivenom that prevented lethality against mice injected with *E.*
380 *romani* (Nigeria) venom, and b) *E. coloratus* and *E. leucogaster* when given the ED₁₀₀ of antivenom
381 that prevented lethality against mice injected with *E. p. leakeyi* venom. ED₅₀ studies initially used four
382 dose groups (the minimum number to determine an ED₅₀ by Probit analysis), and additional dose
383 groups were used if necessary to complete the ED₅₀ curve. ED₅₀ was determined by Probit analysis
384 using Excel (Microsoft, RRID:SCR_016137).

385

386 We used an ED₁₀₀ comparison design for the three additional venoms as opposed to ED₅₀s, which
387 equated to one dose group tested per venom per antivenom, as opposed to at least four dose groups
388 required for ED₅₀. These experiments were designed to provide informative comparative antivenom
389 efficacy readouts with substantially reduced ethical cost via reduction of experimental animal
390 numbers.

391

392 2.10 Role of the funding source

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397 the paper for publication.

398

399 **3. Results**

400 **3.1 Establishing taxonomic identity of captive *Echis* through mitochondrial barcoding**

401 To categorically define if the saw-scaled vipers housed in the herpetarium at LSTM (originating from
402 north-eastern Nigeria), used for EchiTAb antivenom production and considered as *E. ocellatus sensu*
403 *lato*, are *E. ocellatus sensu stricto* or *E. romani*, we implemented a mitochondrial barcoding approach
404 on 40 individuals collected between April 2008 and September 2014. We aligned 789 base pairs (b.p.)
405 of *CYTB* and 644 b.p. of *NADH4* sequence, the aligned fragments corresponding to those used in the
406 previous phylogenetic analysis of the *Echis* genus [6]. The Model function in MEGA 11 identified the
407 Tamura-Nei model [34] with gamma-distributed substitution rates (TN93+G) as the optimal
408 substitution model under the BIC for the data. Phylogenetic analysis of the *NADH4* and *CYTB*
409 sequences amplified from the shed skins or venoms of the 40 individual assumed *E. ocellatus*
410 specimens (from Nigeria) in the LSTM collection demonstrate they are all *E. romani* (Figure 1).
411 Similarly, *NADH4* and *CYTB* sequences amplified from *E. ocellatus* venom originating from Cameroon,
412 sourced from Latoxan, demonstrates clearly it has originated from specimens of *E. romani*, while the
413 sequences amplified from *E. ocellatus* venom originating from Ghana, sourced from Latoxan,
414 demonstrates this venom has originated from specimens of *E. ocellatus sensu stricto* (Figure 1). As a
415 control, we also sequenced *NADH4* and *CYTB* amplicons from DNA extracted from the skin shed of the
416 *E. carinatus* specimen originating from Pakistan, with results confirming its *E. carinatus* designation.
417 All the phylogenetic relationships determined were supported by high bootstrap values (>90). These
418 findings strongly confirm that the clinically trialled and approved EchiTAb antivenoms [9,19] made
419 using venom from Nigerian saw-scaled viper venoms are directed against *E. romani*.

420

421 **3.2 Antivenom recognition of *Echis* venoms by ELISA**

422 We next performed ELISA experiments to quantify the binding levels of each of the three *Echis* specific
423 antivenoms against the various saw-scaled viper venoms. High levels of comparable binding by all
424 three antivenoms (EchiTAbG, SAIMR Echis and Echiven) against the venoms (*E. ocellatus*, *E. romani*
425 [x2], *E. leucogaster*, *E. coloratus* and *E. p. leakeyi*) was observed (Fig. 2A-F). The binding titres remained

426 above naïve control for all antivenoms against all venoms, to at least a 1 in 62,500 dilution of neat
427 antivenom.

428

429 **3.3 Neutralisation of venom PLA₂ activity**

430 The venoms demonstrated considerable differences in enzymatic PLA₂ content, as shown in Fig. 3A (p
431 < 0.0001). The venoms of *E. p. leakeyi* and *E. leucogaster* demonstrated highest activity (19.48 and
432 15.78 [U/mL]/µg respectively). In comparison to *E. p. leakeyi*, the venoms of *E. coloratus* and *E.*
433 *ocellatus* had 3-fold and 5-fold lower PLA₂ activity respectively, and the lowest activity was observed
434 in the two *E. romani* venoms (0.47 and 1.75 [U/mL]/µg for Nigerian and Cameroonian venoms
435 respectively).

436

437 The ability of the three antivenoms to neutralise the PLA₂ activity of these venoms was determined
438 using four different volumes of antivenom (6.25, 3.13, 1.56 and 0.78 µL/well) (Figure 3B-G). All three
439 antivenoms demonstrated strongest neutralisation of PLA₂ activity from Nigerian *E. romani* venom
440 (Figure 3F), with a 95-100% reduction in PLA₂ activity observed at the highest amount of antivenom
441 tested (6.25 µL), and when comparing PLA₂ neutralisation at the lowest volume (0.78 µL), SAIMR Echis
442 showed strongest neutralisation and was significantly more inhibitory than EchiTABG (p = 0.001). The
443 three antivenoms showed comparatively weaker neutralisation of PLA₂ activity from *E. ocellatus*
444 venom and Cameroonian *E. romani* venom (Figure 3D and E). At the lowest volume of antivenom (0.78
445 µL), no significant differences were detected between the antivenoms against *E. ocellatus* (Ghana) (p
446 > 0.8), whereas for *E. romani* (Cameroon) SAIMR Echis was significantly more effective than EchiTABG
447 and Echiven (p < 0.015 for both). All three antivenoms showed comparatively weaker inhibition of the
448 PLA₂ activity of *E. coloratus* venom (Figure 3B), and at the lowest dose of antivenom (0.78 µL) no
449 significant differences between the three antivenoms were observed (p > 0.99). The neutralisation of
450 *E. leucogaster* and *E. p. leakeyi* venom PLA₂ activity showed strong differences between the three test
451 antivenoms. At the lowest dose of antivenom, SAIMR Echis antivenom proved the most effective
452 against both venoms (p < 0.001 compared to EchiTABG and Echiven, for both venoms).

453

454 **3.4 Neutralisation of venom SVMP activity**

455 All *Echis* venoms exhibited strong SVMP activity in the *in vitro* assay compared to PBS control (p <
456 0.001), as shown in Figure 4A. *E. romani* (Nigeria), *E. ocellatus* and *E. p. leakeyi* venoms had
457 comparable SVMP activity (denoted by ^ on Figure 4A), which was significantly greater than the SVMP
458 activity of the *E. romani* (Cameroon), *E. leucogaster* and *E. coloratus* venoms (denoted by # on Figure
459 4A) (p ≤ 0.0009 for all comparisons).

460

461 The ability of the three antivenoms to neutralise the SVMP activity of the six venoms was determined
462 using four different antivenom volumes (2.5, 1.25, 0.625 and 0.313 $\mu\text{L}/\text{well}$) (Figure 4B-G). At 2.5 μL
463 of antivenom, all three antivenoms reduced the SVMP activity of five of the six *Echis* venoms with the
464 exception of *E. p. leakeyi* (Figure 4G) by at least 30%, albeit with large variability between venoms and
465 between antivenoms (Figure 4B-F). At the lowest volume tested (0.313 μL) significant differences
466 between antivenoms only persisted with *E. coloratus* and *E. leucogaster* (Figure 4B and C). For *E.*
467 *coloratus*, SVMP activity was reduced most by SAIMR Echis, although this was not significantly
468 different to EchiTAbG ($p > 0.05$). However, both antivenoms were significantly more effective than
469 Echiven ($p < 0.0001$ for SAIMR Echis and $p = 0.042$ for EchiTAbG). Similarly, SAIMR Echis was also most
470 effective against *E. leucogaster* and this was significantly different to both EchiTAbG ($p < 0.0001$) and
471 Echiven ($p < 0.0001$).

472

473 **3.5 Neutralisation of venom plasma clotting activity**

474 All *Echis* venoms possessed a significant ability to cause clotting of bovine plasma in the *in vitro* assay
475 compared to PBS control ($p < 0.0001$), and five of the venoms had comparable activity whilst the
476 clotting effect of *E. leucogaster* was significantly higher ($p = 0.002$, denoted by \wedge) (Figure 5A).

477

478 The same four volumes of antivenom as those used in the SVMP assay (2.5, 1.25, 0.625 and 0.313
479 $\mu\text{L}/\text{well}$) were tested to determine their ability to also neutralise *Echis* venom clotting capabilities, as
480 shown in Figure 5B-G. Similar to the SVMP assay, 2.5 μL of each antivenom was able to reduce clotting
481 activity against all six *Echis* venoms. However, the percentage of clotting activity remaining following
482 coincubation with the antivenoms at 2.5 μL was variable across the venoms and antivenoms. Whilst
483 SAIMR Echis and Echiven were able to reduce coagulopathic activity to approximately 50% for the
484 majority of venoms (with the exception of Echiven against *E. coloratus*), EchiTAbG could only achieve
485 this against *E. ocellatus*, *E. romani* (Cameroon) and *E. leucogaster*.

486

487 At the lowest volume of antivenom tested (0.313 μL), there were significant differences between the
488 effectiveness of the antivenoms to modulate clotting activity. Within the *E. ocellatus* and *E. romani*
489 venoms, SAIMR Echis was significantly better at reducing the clotting activity of the Nigerian locality
490 than Echiven or EchiTAbG (reduced to $< 50\%$ vs 70-90% respectively, $p < 0.0001$) (Figure 5F), whilst
491 against the Cameroon locality (Figure 5E) and *E. ocellatus* (Figure 5D), SAIMR Echis and Echiven were
492 both significantly better than EchiTAbG ($\sim 50\%$ compared to $\sim 70\%$, $p < 0.0001$). This pattern also
493 persisted for the antivenoms against *E. leucogaster* (Figure 5C), although all three antivenoms were

494 less effective at reducing clotting activity than against the Cameroon *E. romani* and Ghana *E. ocellatus*
 495 venoms with the same volume of antivenom. Against *E. coloratus* and *E. p. leakeyi* (Figure 5B and G)
 496 SAIMR Echis was again significantly better than the other two antivenoms ($p < 0.0001$) and reduced
 497 clotting activity by over 20% more than either Echiven or EchiTABG. However, whilst there was no
 498 difference between Echiven or EchiTABG against *E. coloratus*, Echiven was still significantly better than
 499 EchiTABG at reducing clotting caused by *E. p. leakeyi* venom (75% vs 88% activity remaining, $p =$
 500 0.0001).

501

502 3.6 Ability of antivenoms to neutralise murine venom induced lethality

503 The volume of antivenom that prevented lethality in 50% of animals when challenged with 5 X LD₅₀ of
 504 venom (ED₅₀) was determined for the three antivenoms against *E. romani* (Nigeria) and *E. p. leakeyi*
 505 (Figure 6). The ED₅₀s, in a range of metrics including volume (μL), the WHO-recommended metric of
 506 Potency [35], and in μL/mg [40], are presented in Table 3 and Figure 6A-C. LD₅₀ values available in the
 507 literature were used for preparing 5 x LD₅₀ doses for each of the *Echis* venoms (as shown in Table 2).
 508 ED₅₀ experiments demonstrated stark differences in the amount of antivenom required to neutralise
 509 50% of lethality. By volume of antivenom, SAIMR Echis was the most potent antivenom against both
 510 venoms (Fig. 6A), whilst for Echiven approximately two-fold more antivenom was required. The ED₅₀
 511 for EchiTABG required >7-fold more volume of antivenom for *E. romani* (Nigeria) and >12-fold
 512 antivenom for *E. p. leakeyi*, when compared to SAIMR Echis. Similarly, when reported as potency,
 513 where larger values indicate higher potency, SAIMR Echis was the most potent against both *E. romani*
 514 (Nigeria) and *E. p. leakeyi* (Fig. 6B). The potency of SAIMR Echis was more than 2-fold greater than
 515 that of Echiven against both venoms, and was 7-fold and 13-fold greater than EchiTABG against *E.*
 516 *romani* (Nigeria) and *E. p. leakeyi* respectively. Similar trends were observed for the other metrics of
 517 efficacy.

518

519 **Table 3. Antivenom efficacy against *E. romani* (Nigeria) and *E. p. leakeyi* in a murine pre-incubation**
 520 **model.** The efficacy of EchiTABG, SAIMR Echis and Echiven against *E. romani* (Nigeria) and *E. p. leakeyi*
 521 reported as ED₅₀ (μL), potency (n-1 LD₅₀ / ED₅₀) and volume per mg of venom (μL/mg). 95% confidence
 522 intervals indicated in parentheses.

<i>E. romani</i> (Nigeria)	EchiTABG	SAIMR Echis	Echiven
ED₅₀ (μL)	50.06 (35.08 – 71.45)	7.07 (5.47 – 9.14)	15.00 (10.05 – 22.39)
Potency	0.08 (0.06 – 0.11)	0.57 (0.44 – 0.73)	0.27 (0.18 – 0.40)
μL/mg	560.90 (393.05 – 800.56)	79.22 (61.29 – 102.41)	168.07 (112.61 – 250.87)

<i>E. p. leakeyi</i>	EchiTABG	SAIMR Echis	Echiven
ED ₅₀ (μL)	89.31 (64.71 – 123.27)	7.30 (3.68 – 14.49)	20.42 (14.26 – 29.23)
Potency	0.04 (0.03 – 0.06)	0.55 (0.28 – 1.09)	0.20 (0.14 – 0.28)
μL/mg	1318.23 (955.13 – 1819.48)	107.75 (54.32 – 213.87)	301.40 (210.48 – 431.44)

523

524 We next implemented a comparative dose assay, similar to one previously utilised for comparing the
 525 efficacy of various polyvalent antivenoms for East Africa [24], rather than performing full ED₅₀, to
 526 reduce the quantity of mice required for comparative testing of each antivenom against each
 527 additional venom. Using the ED₁₀₀ dose of each antivenom (the volume of antivenom which conferred
 528 100% protection in experiments to obtain antivenom ED₅₀ values) we examined each antivenom's
 529 ability to neutralise 5 x LD₅₀s of *E. romani* (Cameroon) and *E. ocellatus* venoms (using ED₁₀₀s
 530 determined against Nigerian *E. romani*) or *E. leucogaster* and *E. coloratus* venoms (using ED₁₀₀s
 531 determined against *E. p. leakeyi*). Of note, the reported ED₁₀₀ volume for EchiTABG only conferred 80%
 532 protection (100% protection was not achieved in the ED₅₀ experiment).

533

534 **Table 4. Antivenom volume to confer 100% survival (ED₁₀₀) against *E. romani* (Nigeria) and *E. p.*
 535 *leakeyi* in a murine pre-incubation model.** The ED₁₀₀ (volume of antivenom that conferred 100%
 536 survival) of the test antivenoms EchiTABG, SAIMR Echis and Echiven against *E. romani* (Nigeria) and *E.*
 537 *p. leakeyi* in the pre-incubation model of envenoming. All assays used a venom challenge dose of 5 x
 538 LD₅₀ (Table 2).

Venom	EchiTABG	SAIMR Echis	Echiven
<i>E. romani</i> (Nigeria)	100 μL	25 μL	30 μL
<i>E. p. leakeyi</i>	90 μL	50 μL	60 μL

539

540 When using the ED₁₀₀ doses (ED₁₀₀ dose observed for *E. romani* [Nigeria], Table 4) of each antivenom
 541 against 5 x LD₅₀ of *E. romani* (Cameroon), SAIMR Echis and Echiven provided partial protection at the
 542 end of experiment (80% survival and 40% survival, respectively and mean survival times 314 minutes
 543 and 201 minutes, respectively) (Figure 7C and 7E), while the mean survival time for EchiTABG was 99
 544 minutes (20% survival). When using the same ED₁₀₀ doses against 5 x LD₅₀ of *E. ocellatus* (Ghana), both
 545 SAIMR Echis and Echiven provided complete protection, with 100% of mice surviving until the end of
 546 experiment. In contrast, EchiTABG failed to prevent lethality but further increased time to humane
 547 endpoint (mean survival times 95 minutes, compared to 6 minutes with no antivenom). Challenging
 548 mice with 5 x LD₅₀ of *E. coloratus* venom preincubated with the ED₁₀₀ dose for each antivenom (the
 549 ED₁₀₀ dose observed for *E. p. leakeyi*, Table 4) resulted in 0% survival with EchiTABG and Echiven, and

550 20% survival with SAIMR Echis (Figure 7A). In contrast, using the same ED₁₀₀ doses, SAIMR Echis and
551 Echiven performed well against 5 x LD₅₀ of *E. leucogaster* venom (conferring 100% and 80% survival
552 respectively at 6 hours), whilst EchiTABG failed to confer full protection against venom-induced
553 lethality but increased mean survival times to 124.8 minutes from 7.8 minutes without antivenom
554 (Figure 7B and 7E).

555

556 Discussion

557 Given the medical significance of envenoming by snakes of the genus *Echis* [36], the identification of
558 efficacious antivenoms suitable to treat such snakebites is integral to the WHO's objectives to halve
559 snakebite mortality and morbidity by 2030 [4]. This preclinical study aimed to test available *Echis*-
560 specific monospecific antivenoms and directly compare their ability to neutralise *Echis* venoms from
561 multiple localities and species both *in vitro* and in preclinical murine models of envenoming. This is
562 particularly important considering the recent changes in taxonomy to the genus which has seen *E.*
563 *ocellatus*, historically viewed as the most medically important species of the genus in sub-Saharan
564 Africa [10], split into *E. romani* and *E. ocellatus* [11]. The recent taxonomic change raised important
565 questions regarding differences in venom composition between the newly identified *E. romani* and *E.*
566 *ocellatus*, and raised uncertainty around potential efficacy of antivenoms indicated for *E. ocellatus*
567 pre-species partition, which we sought to address in this study. The saw-scaled vipers housed in
568 LSTM's herpetarium that were barcoded in this study all originate from the Kaltungo (Gombe) region
569 of north-eastern Nigeria. The barcoding results presented here clearly demonstrate that all these
570 animals, historically considered *E. ocellatus* prior to partition, are *E. romani*, further evidencing the
571 apparent distinct geographical ranges of the newly partitioned species [6,11]. Furthermore, based on
572 the genetic barcoding results presented, it is likely that several existing antivenoms indicated for *E.*
573 *ocellatus* envenoming are highly likely to have been manufactured using a mixture of *E. romani* and *E.*
574 *ocellatus* venom or solely *E. romani* venom. The latter will certainly be the case with EchiTABG and
575 the trivalent antivenom EchiTAB-Plus-ICP, which have been manufactured using the venom of some
576 of the snakes barcoded in this study [19].

577

578 Despite the partition of *E. ocellatus* occurring in 2018, it was not until recently that the new species
579 designations started to appear in scientific literature. To maximise the reproducibility of results in
580 toxinology and standardise antivenoms and other treatments, we urge other toxinologists working on
581 the *E. ocellatus* complex to pay close attention to the origins of snakes/venoms and the affinities of
582 antivenoms involved, and to use up-to-date nomenclature in their publications, as previously outlined
583 [37,38]. With the confirmation of the identity of venom from both *E. romani* and *E. ocellatus*, we

584 compared the potential of three *Echis* monospecific antivenoms, EchiTABG, SAIMR Echis and Echiven,
585 to neutralise the *in vitro* and *in vivo* toxin activities of each venom and of venom from a further three
586 *Echis* species (*E. coloratus*, *E. leucogaster* and *E. p. leakeyi*).

587

588 The main proteinaceous components of *Echis* venoms are SVMPs, PLA₂, C-type lectin-like proteins,
589 serine proteases, disintegrins, and L-amino acid oxidases [17,21,39]. In particular, the *Echis* genus of
590 snakes have a remarkably high abundance of SVMPs but with substantial variations in quantity
591 observed between individual species; SVMPs comprise up to 70% of *E. ocellatus/romani* venom, whilst
592 *E. leucogaster* contains only 27% [40], and this variation was reflected in *in vitro* SVMP activity. These
593 zinc dependent proteinases play a fundamental role in driving life-threatening venom-induced
594 consumption coagulopathy and systemic haemorrhage [41]. The *in vitro* SVMP activity of each of the
595 six venoms were significantly neutralised by the highest dose of each antivenom, however, it must be
596 noted that SAIMR Echis and EchiTABG performed poorest against *E. p. leakeyi* venom in comparison
597 to the other venoms. The failure or poor *in vitro* neutralisation of the SVMP activity of *E. p. leakeyi* at
598 the highest dose tested may reflect the relatively high abundance of SVMPs belonging to the PII family
599 in *E. p. leakeyi* venom compared to the low abundance of PII SVMPs in the venoms of *E.*
600 *ocellatus/romani* against which the antivenoms were raised. A previous transcriptome analysis of
601 various *Echis* species [42] demonstrated proportional differences in relative Group II PLA₂ abundance,
602 with *E. p. leakeyi* containing the highest expression of PLA₂ transcripts and markedly less detected in
603 *E. romani* (previously *E. ocellatus*) and *E. coloratus* [42]. Similarly, venom analyses of *E. romani*
604 (previously *E. ocellatus*) from different locales demonstrated intraspecies differences in the
605 abundance of PLA₂ [43], and this was demonstrated by evident differences observed in our *in vitro*
606 PLA₂ assay. Bearing in mind the known variation in venom toxins and subsequent activity, this
607 reiterates the importance of carefully evaluating the source of venoms used for antivenom production
608 and the need for thorough and transparent preclinical testing of proposed species efficacy.

609

610 Whilst *in vitro* assays are an important tool to identify potential efficacious snakebite treatments and
611 define their neutralisation capabilities against specific toxin families, they have numerous limitations
612 including restriction to specific subclasses and forms within toxin families. Preclinical efficacy
613 recommendations remain heavily reliant on murine neutralisation of lethality assays due to the
614 complexity and multiplicity of venom activities *in vivo*. The *in vivo* efficacy results presented here
615 demonstrate that each *Echis* monospecific antivenom was capable of neutralising the lethal effects of
616 *E. p. leakeyi* and Nigerian *E. romani*, however notable differences are seen in their comparative
617 potency against venoms from *E. ocellatus* and other geographic locales of *E. romani*, and this may

618 have implications for their clinical use. The calculated ED₅₀ values were in broad agreement to
619 previously calculated ED₅₀ values for EchiTabG and SAIMR Echis against Nigerian *E. romani* (formerly
620 *E. ocellatus*) venom and SAIMR Echis for *E. p. leakeyi* venom [22], with SAIMR Echis possessing the
621 most potent venom-neutralising ability (lowest ED₅₀ and highest potency value) against both *E. romani*
622 (Nigeria) and *E. p. leakeyi*. In comparison, Echiven was two-fold less potent against both venoms and
623 EchiTabG was 7-fold and 13-fold less potent against the venoms, respectively. When accounting for
624 total protein concentration of the three *Echis* monospecific antivenoms (Table 1), the differences in
625 neutralising efficacy between products were more modest, although the trends in neutralising ability
626 remained.

627
628 When the antivenoms were assessed further for cross-reactivity, intra-country and intra-species
629 differences became apparent. The dose of each antivenom that neutralised 100% of lethality against
630 *E. romani* (Nigeria) was unable to fully protect mice challenged with *E. romani* venom from Cameroon,
631 whilst SAIMR Echis and Echiven fully prevented lethality from *E. ocellatus* but EchiTabG failed to
632 prevent lethality from this venom. We believe these differences in efficacy could be explained by
633 different challenge doses used for the different venoms, but further investigation such as full ED₅₀
634 experiments would be useful to identify whether the antivenoms can provide full protection. Similarly,
635 SAIMR Echis and Echiven showed strong cross-reactivity with *E. leucogaster*, whilst EchiTabG failed to
636 prevent lethality from this venom. In contrast to previous studies that demonstrated EchiTabG could
637 neutralise the lethal effects of *E. coloratus* [24], in our study all three antivenoms were ineffective
638 against this venom. A larger volume of EchiTabG was used than the previous study, and the same
639 challenge dose of venom was used in both, so these findings were unexpected and could potentially
640 be due to batch variation of either the challenge venom or the antivenom. Although the three
641 antivenoms in this study lacked efficacy against Egyptian *E. coloratus* venom, it would be worth
642 confirming that the commercial *E. coloratus* antivenom produced in Israel [44,45] has good efficacy
643 against this venom to ensure adequate treatments against bites from this species.

644
645 To uphold principles of the 3Rs (replacement, refinement and reduction of animals in research), we
646 used a comparator model of envenoming [24] which enables prediction of antivenom performance
647 but requires fewer mice than full ED₅₀ testing. We challenged mice with either the venom of *E. romani*
648 (Cameroon) and *E. ocellatus*, or *E. leucogaster* and *E. coloratus*, with corresponding antivenom doses
649 which provided 100% protection against *E. romani* (Nigeria) and *E. p. leakeyi*, respectively. The results
650 of the ED₁₀₀ comparator assays mirrored the *in vitro* and ED₅₀ findings, with substantial differences in
651 dose-matched potency of antivenoms against other species. The most notable and perhaps

652 unexpected finding was the reduced or poor ability of antivenoms to protect mice from *E. romani*
653 (Cameroon) envenoming when using the ED₁₀₀ *E. romani* (Nigeria) antivenom dose, with 60% and 80%
654 of mice succumbing to venom effects when dosed with Echiven and EchiTAbG, respectively. This
655 suggests that *E. romani* venoms from different localities have different potencies and thus differences
656 in their ability to be neutralised by antivenoms, meriting further research to understand the impact
657 of intraspecific *E. romani* venom variation on antivenom efficacy. This illustrates how, given the
658 frequency of sometimes extreme venom variation within species, even in the face of extensive gene
659 flow [46], taxonomic revisions should be seen as broad roadmaps for additional research into
660 antivenom efficacy, but not interpreted as robust predictors of venom composition or antivenom
661 effectiveness [47]. In view of the public health importance of the *E. ocellatus* complex, further
662 research into variation in venom composition within the group would be advisable. It is also important
663 to keep in context here that only a single dose has been examined to enable comparative analysis of
664 an antivenom's ability to neutralise different venoms at that dose. The failure of an antivenom to
665 protect mice from envenoming in these experiments, while it is indicative of potential potency of an
666 antivenom, it is not capable of definitively saying if an antivenom is ineffective, and results should
667 therefore be viewed in this context and treated with caution.

668
669 EchiTAbG has been proven to be clinically effective in Nigeria [9,18,19], and has a WHO positive risk-
670 benefit assessment for treatment against *E. ocellatus* (although it is not clear if this now corresponds
671 to *E. ocellatus* and *E. romani*, or the historical *E. ocellatus* complex) and *E. pyramidum* in a broad range
672 of countries [48]. Whilst previous products produced by VINS for use in Africa have not proven
673 efficacious in independent testing [24,43] our murine model of systemic envenoming demonstrated
674 both SAIMR Echis and the sample of Echiven provided by VINS had relative superior dose efficacy for
675 all venoms investigated compared to EchiTAbG. We hope this study provides the requisite preclinical
676 evidence to support the transition of suitable Echis monospecific antivenoms into clinical trials to
677 assess their clinical efficacy against African saw-scaled viper envenoming. Such clinical efficacy testing
678 would ideally be in a randomised clinical trial, also incorporating EchiTAbG, performed in distinct parts
679 of the African continent against a variety of geographically- and species-distinct *Echis* envenomings.

680
681 In summary, all antivenoms conveyed a degree of intra-genus pre-clinical neutralisation amongst the
682 sub-Saharan African *Echis* venoms, although this was highly variable across the different *Echis* species
683 and the three *Echis* monospecific antivenoms tested. All antivenoms performed poorly against *E.*
684 *coloratus*, suggesting that antivenoms designed for sub-Saharan African *Echis* spp. will have little to
685 no preclinical efficacy against *Echis* species out of this geographic range. Further research is needed
686 to ascertain efficacy of other medically important African *Echis* species, including *E. jingeri*. Ultimately,

687 our data provides the first empirical evidence of differences in venom potencies and antivenom
688 efficacies against the recently partitioned medically important west African saw-scaled viper species
689 *E. romani* and *E. ocellatus*.

690

691 **Contributors**

692 Conceptualisation: SKM, NRC, SA

693 Data curation: RJE, MK, MCW, TX, SKM

694 Formal analysis: RJE, MK, MCW, TX, SKM, WW

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699 Resources: WW, EPC, SKM

700 Writing – original draft: RJE, WW, NRC, SRA, SKM

701 Writing – review and editing: All authors

702

703 **Declaration of Interests**

704 SKM and NRC communicated with the antivenom manufacturer VINS Bioproducts to obtain a sample
705 of Echiven antivenom for testing. The antivenom manufacturer had no role in the study design, data
706 collection and analysis, decision to publish or preparation of the manuscript. The Centre for Snakebite
707 Research and Interventions (CSRI) at LSTM was historically involved in the development of EchiTABG
708 and EchiTAB-Plus0ICP antivenoms, though none of the authors from CSRI were directly involved in this
709 work. NRC was previously employed by the manufacturer of EchiTABG antivenom (MicroPharm, UK)
710 between 2010 and 2012. NRC and CAD are currently collaborators of the EchiTABG manufacturer
711 MicroPharm, UK. Micropharm had no role in the study design, data collection and analysis, decision
712 to publish or preparation of the manuscript.

713

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722

723 **Data sharing**

724 All supporting data are available without restrictions in the Supplementary Data files. DNA sequences
725 are available in GENBANK (RRID:SCR_002760) under accession numbers (OQ735307-OQ735376).

726

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879 [outcomes](https://extranet.who.int/prequal/vaccines/list-product-assessment-outcomes)

880

881

882

883 **Figure Legends**

884 **Figure 1. Maximum likelihood phylogeny of the *Echis ocellatus* group inferred from 1433 base pairs**
885 **of mitochondrial CYTB and NADH4 sequence.** Node labels next to major nodes represent %bootstrap
886 support.

887

888 **Figure 2. The titre of three antivenoms against six *Echis* venoms determined by end-point titration**
889 **ELISA.** EchiTABG shown in magenta, SAIMR Echis shown in teal, Echiven shown in blue. Venom-naïve
890 equine F(ab)₂ shown in black and venom-naïve ovine IgG shown in purple. Panel a: *E. coloratus*. Panel
891 b: *E. leucogaster*. Panel c: *E. ocellatus* (Ghana). Panel d: *E. romani* (Cameroon). Panel e: *E. romani*
892 (Nigeria). Panel f: *E. p. leakeyi*. Data points represent the mean of two replicates and error bars show
893 the standard deviation.

894

895 **Figure 3. PLA₂ activity of six *Echis* venoms and their neutralisation by the three different antivenoms.**
896 a: PLA₂ activity of *E. romani* (CAM = Cameroon and NGA = Nigeria), *E. ocellatus* (GHA = Ghana), *E.*
897 *coloratus*, *E. leucogaster* and *E. p. leakeyi*. Samples were subtracted for background and converted to
898 activity in (U/mL)/μg by extrapolation from a bee venom standard curve. Data show the mean of three
899 replicates and error bars represent standard deviation. Statistical differences in activity compared to
900 'buffer only' were determined by one-way ANOVA, with venom specific p values indicated above the
901 bar (ns = not significant, p > 0.05).

902 b-g: Neutralisation of (b) *E. coloratus*, (c) *E. leucogaster*, (d) *E. ocellatus* (Ghana), (e) *E. romani*
903 (Cameroon), (f) *E. romani* (Nigeria), (g) *E. p. leakeyi* PLA₂ activity by the three antivenoms EchiTABG
904 (ETG), SAIMR Echis (SE) and Echiven (EV) at different doses, expressed as a percentage of a no
905 antivenom control showing 100% activity. Data show the mean of three replicates and error bars
906 represent standard deviation. Two-way ANOVA was performed to compare differences in PLA₂ activity
907 at the 0.78 μL dose of antivenoms. * indicates p < 0.05, **** indicates p < 0.001, ns = not significant
908 (p > 0.05).

909

910 **Figure 4. SVMP activity of six *Echis* venoms and their neutralisation by the three different**
911 **antivenoms.**

912 a: SVMP activity of *E. romani* (CAM = Cameroon and NGA = Nigeria), *E. ocellatus* (GHA = Ghana), *E.*
913 *coloratus*, *E. leucogaster* and *E. p. leakeyi*. Data show the mean of four replicates and error bars
914 represent standard deviation. Statistical differences in activity compared to PBS, and between

915 venoms, were determined by one-way ANOVA, with p values against PBS indicated above the bar, and
916 # indicating the three venoms with significantly lower activity and ^ indicating the three venoms with
917 significantly higher activity.

918 b-g: Neutralisation of (b) *E. coloratus*, (c) *E. leucogaster*, (d) *E. ocellatus* (Ghana), (e) *E. romani*
919 (Cameroon), (f) *E. romani* (Nigeria), (g) *E. p. leakeyi* SVMP activity by the three antivenoms EchiTabG
920 (ETG), SAIMR Echis (SE) and Echiven (EV) at different doses, expressed as a percentage of a no
921 antivenom control showing 100% activity. Data show the mean of four replicates and error bars
922 represent standard deviation. One-way ANOVA was performed to compare differences in SVMP
923 activity at the 0.31 μ L dose of antivenoms. * indicates $p < 0.05$, **** indicates $p < 0.001$, ns = not
924 significant ($p > 0.05$).

925

926 **Figure 5. Plasma clotting activity of six *Echis* venoms and their neutralisation by the three different**
927 **antivenoms.**

928 a: Plasma clotting activity of *E. romani* (CAM = Cameroon and NGA = Nigeria), *E. ocellatus* (GHA =
929 Ghana), *E. coloratus*, *E. leucogaster* and *E. p. leakeyi* Data show the mean of four replicates and error
930 bars represent standard deviation. Statistical differences in activity compared to PBS, and between
931 venoms, were determined by one-way ANOVA, with p values against PBS indicated above the bar, and
932 ^ indicating *E. leucogaster* had significantly higher clotting activity compared to the other five venoms.

933 b-g: Neutralisation of (b) *E. coloratus*, (c) *E. leucogaster*, (d) *E. ocellatus* (Ghana), (e) *E. romani*
934 (Cameroon), (f) *E. romani* (Nigeria), (g) *E. p. leakeyi* plasma clotting activity by the three antivenoms
935 EchiTabG (ETG), SAIMR Echis (SE) and Echiven (EV) at different doses, expressed as a percentage of a
936 no antivenom control showing 100% activity. Data show the mean of four replicates and error bars
937 represent standard deviation. One-way ANOVA was performed to compare differences in plasma
938 clotting activity at the 0.31 μ L dose of antivenoms. **** indicates $p < 0.001$, ns = not significant ($p >$
939 0.05).

940

941 **Figure 6. ED₅₀ of the three antivenoms against *E. romani* (Nigeria) and *E. p. leakeyi* against 5 x LD₅₀**
942 **venom dose.**

943 ED₅₀ is defined as the volume of antivenom which protects 50% of mice from the lethal effects of
944 venom. Each experiment used four to five mice per dose group (exact numbers indicated in Supp. File
945 6), challenged with a dose of 5 x venom LD₅₀s and monitored for 6 hours. EchiTabG shown in black,
946 SAIMR Echis shown in magenta, Echiven shown in teal. ED₅₀ was determined using Probit analysis,
947 data represents the calculated ED₅₀ and error bars represent 95% confidence intervals. a: ED₅₀

948 reported in volume (μL of antivenom). b: Potency, where potency is calculated as $(n-1 \text{ LD}_{50})/\text{ED}_{50}$. c:
949 ED_{50} reported in μL of antivenom per mg of venom.

950

951 **Figure 7. Efficacy of the three antivenoms tested at a single dose (ED_{100}) against *E. coloratus*, *E.*
952 *leucogaster*, *E. romani* (Cameroon) and *E. ocellatus* (Ghana).**

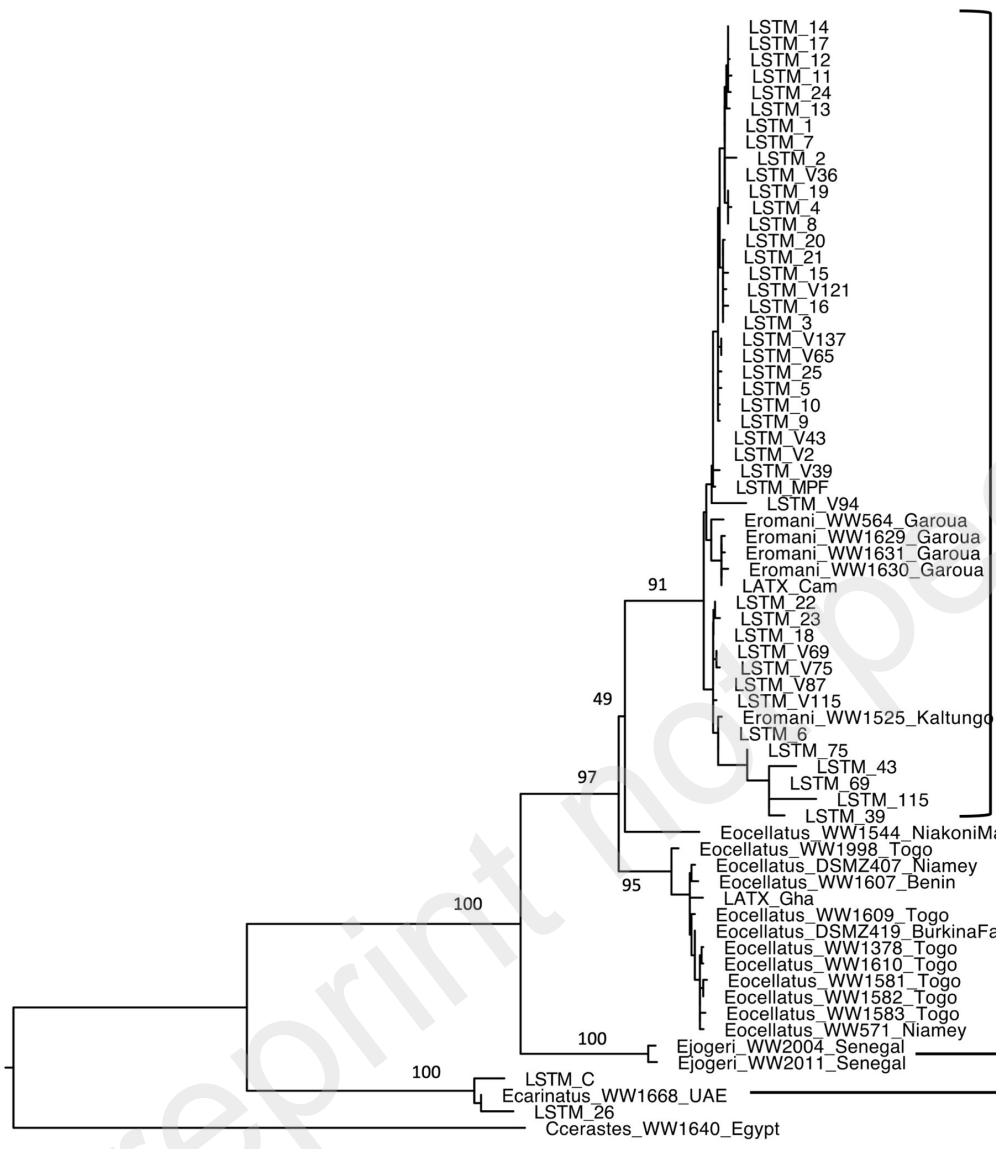
953 a: *E. coloratus* b: *E. leucogaster* c: *E. romani* (Cameroon) d: *E. ocellatus* (Ghana). e: Mean survival time
954 of animals – bars indicate mean survival time and markers indicate individual survival times for each
955 animal. Each experiment used five mice per dose group, challenged with a dose of 5 x venom LD_{50} s
956 and monitored for 6 hours. EchiTABG shown in black, SAIMR Echis shown in magenta, Echiven shown
957 in teal.

958

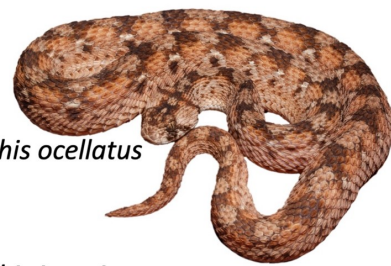
959 **Supplemental Figure 1. PLA_2 assay optimisation.** Graphs show the fluorescence intensity measured
960 in the EnzCheck PLA_2 assay with different amounts of each venom. Amounts of venom that fall in the
961 linear range were used for subsequent assays of venom PLA_2 neutralisation by antivenoms.

962 a: *E. coloratus*, b: *E. leucogaster*, c: *E. romani* (Cameroon), d: *E. ocellatus* (Ghana), e: *E. romani* (Nigeria)
963 and f: *E. p. leakeyi*. Samples were subtracted for background fluorescence. Data show the mean of
964 three replicates and error bars represent standard deviation.

965



Echis romani



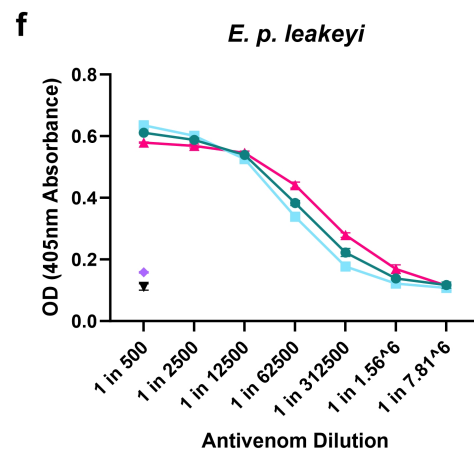
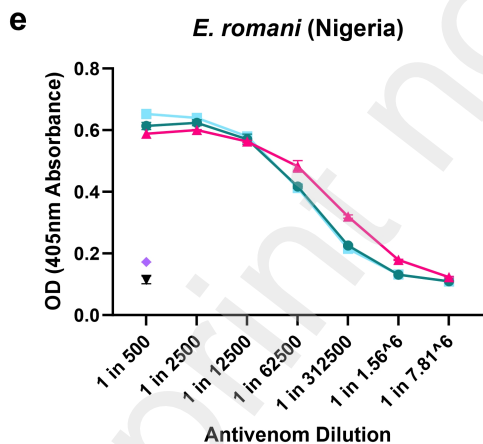
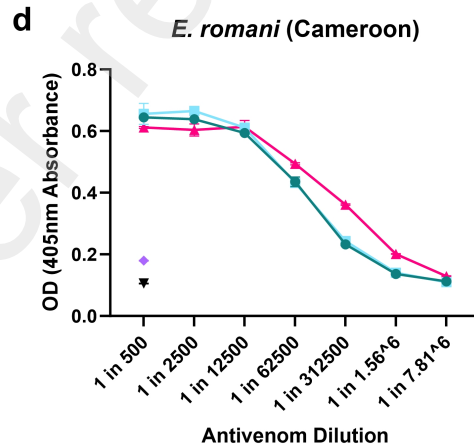
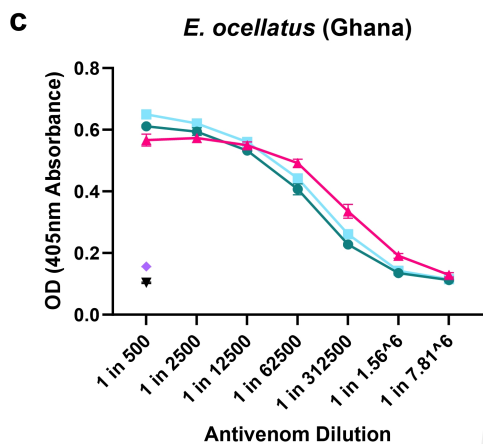
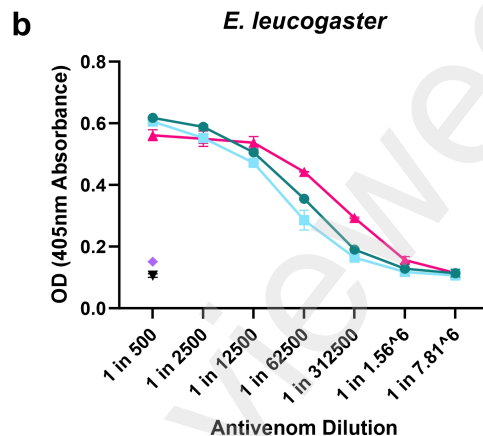
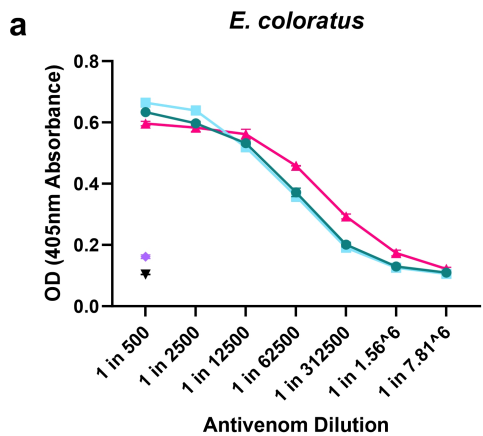
Echis ocellatus

Echis jogeri

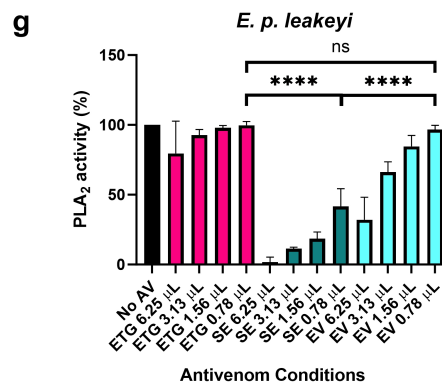
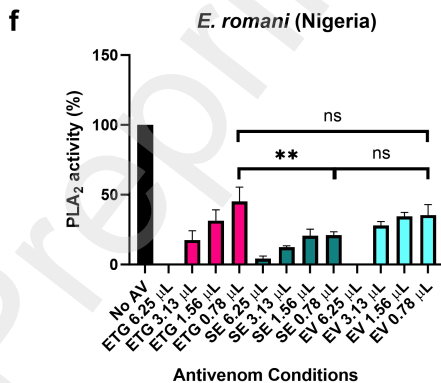
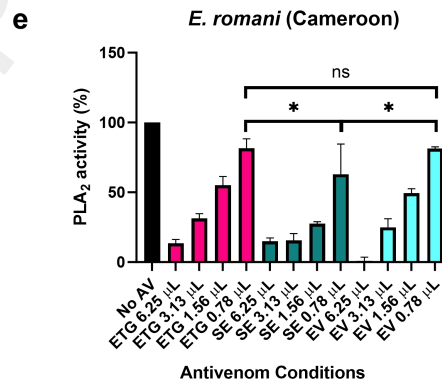
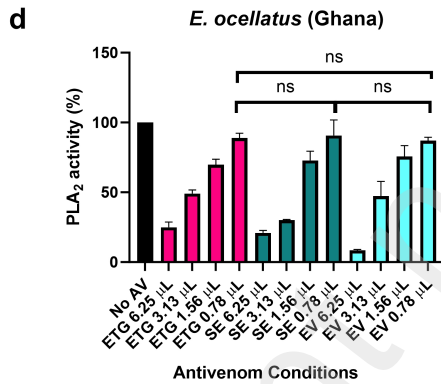
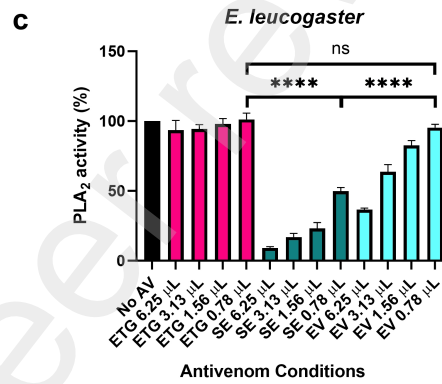
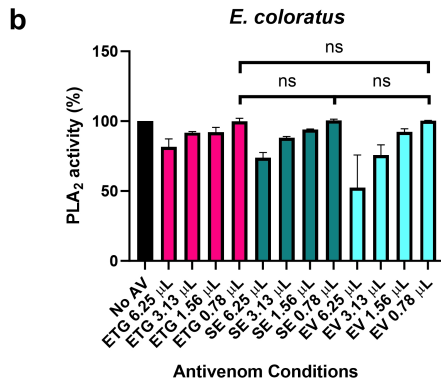
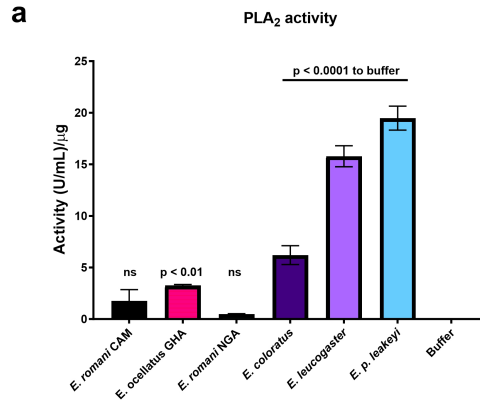
Echis carinatus

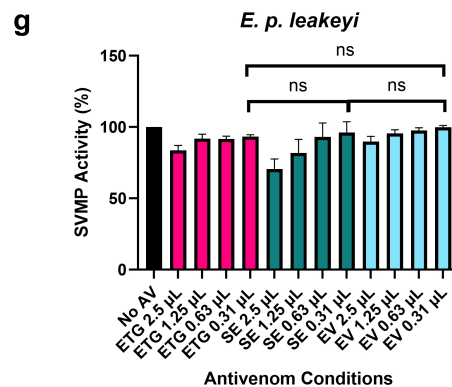
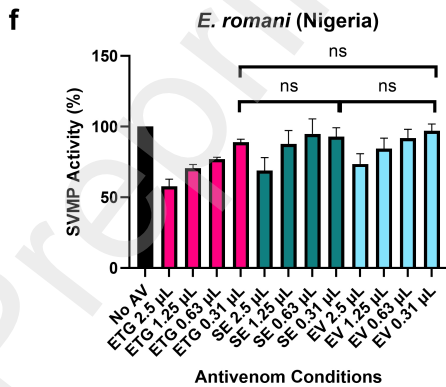
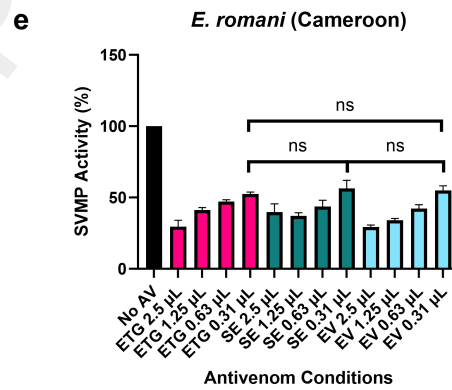
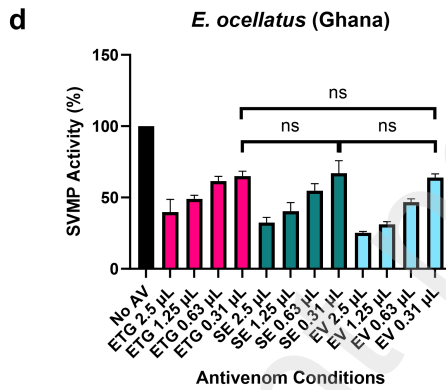
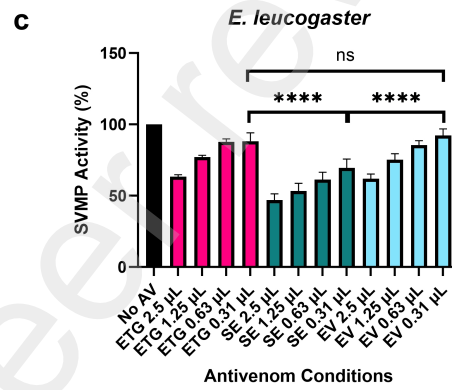
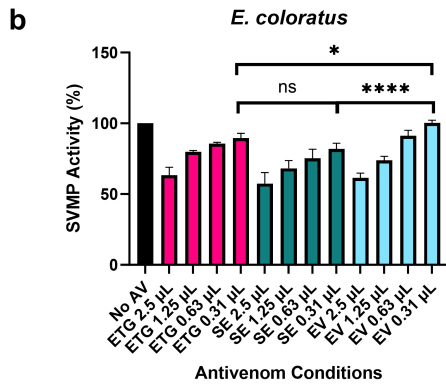
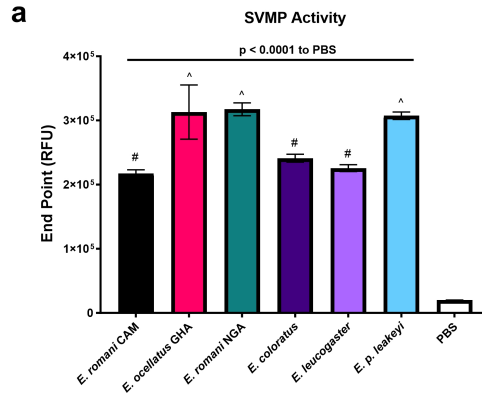
C. cerastes

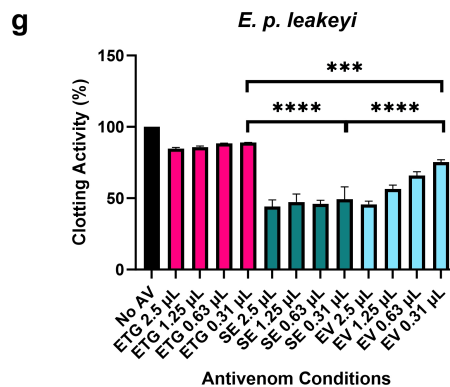
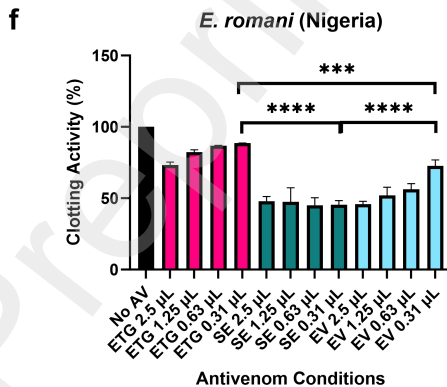
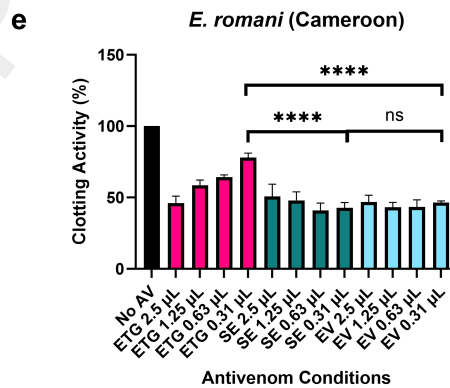
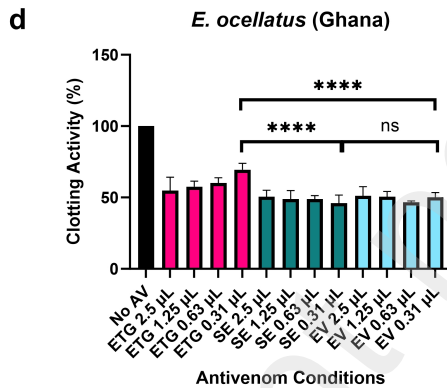
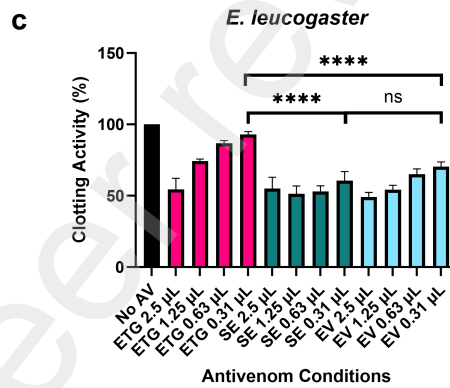
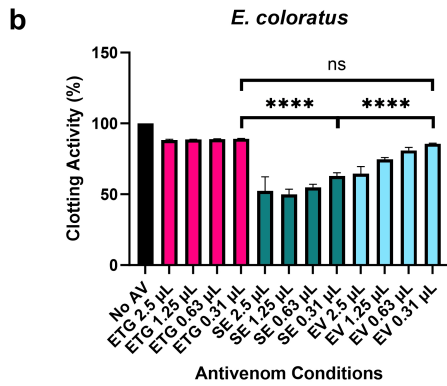
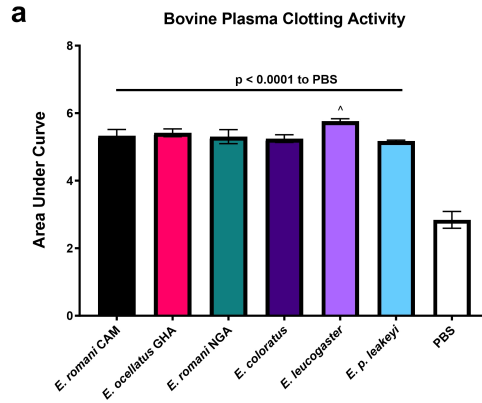
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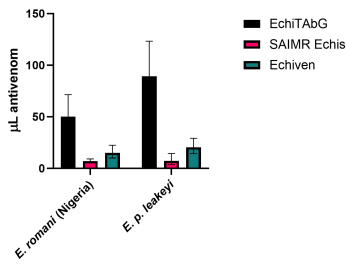
▲ EchiTABG
 ● SAIMR Echis
 ■ Echiven
▼ Naive equine F(ab')₂
 ◆ Naive ovine IgG



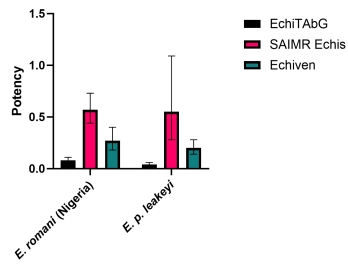




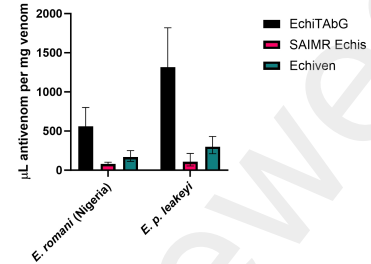
a ED50 (μL antivenom)



b Potency



c ED50 (μL per mg)



Preprint not peer reviewed

