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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Full title: 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*

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

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Research in Context

Evidence before this study

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

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

Added value of this study

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

Implications of all the available evidence

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

Abstract

Background

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

Methods

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

Findings

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

Interpretation

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

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1. Introduction

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

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

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

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

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

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

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

2. Methods

2.1 Venoms

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

2.2 Total DNA extraction and Sanger sequencing

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

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

2.3 Phylogenetic analysis

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

2.4 Antivenoms and control immunoglobulins

Antivenoms used were; (i) EchiTAbG (whole ovine IgG manufactured by MicroPharm UK Ltd) raised against saw-scaled viper venom of Nigerian origin classified at the time as *E. ocellatus* (venom provided by LSTM), (ii) snake venom antiserum (Echis) "Echiven" (equine F[ab]'$_2$ manufactured by VINS Bioproducts Ltd, India) raised against the venom of saw-scaled vipers from Cameroon, Ghana and Mali (provided by Latoxan, France, all listed as *E. ocellatus*) and (iii) "SAIMR Echis carinatus" antivenom (equine F[ab]'$_2$ manufactured by South African Vaccine Producers PTY, South Africa) raised against *E.*

ocellatus of unknown geographical origin. Note that all African saw-scaled vipers were formerly included in *E. carinatus* prior to multiple taxonomic revisions – *E. carinatus* now refers solely to the species *E. carinatus*, found from the United Arab Emirates, Iraq and Turkmenistan south-east to India and Sri Lanka, while African *Echis* were split into several new species [6] and *E. ocellatus* has since been split into *E. ocellatus* and *E. romani*. Antivenoms EchiTAbG and SAIMR Echis were donated to Liverpool School of Tropical Medicine by UK health authorities post expiry, whilst a sample of Echiven was kindly donated by VINS Bioproducts Ltd. The lyophilised Echiven was resuspended in 10 mL sterile water (provided by the manufacturer) prior to use. Control equine $F(ab)'_2$ for incorporation as a control in the *in vitro* experiments described below was produced from equine IgG (BioRad #PEP001) using the Pierce $F(ab)'_2$ Preparation Kit (Pierce, ThermoScientific #44988) according to manufacturer's protocols.

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

Table 1. An overview of the antivenoms used in this study. The table shows manufacturer information, batch/lot numbers and expiry dates, composition of antivenom immunoglobulins and protein concentration and standard deviation as determined by BCA assay, and the venoms against which the manufacturers claim the antivenoms are effective. The asterisk denotes what is indicated 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

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, *E. ocellatus**
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 *E. carinatus* / ocellatus* and paraspecific against *E. coloratus* and two species of *Cerastes*
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	*E. ocellatus**

2.5 End-point ELISA

Venoms from six *Echis* species (*E.* coloratus, *E. leucogaster*, *E. ocellatus* [Ghana], *E. romani* [Nigeria], *E. romani* [Cameroon], *E. p. leakeyi*) were coated at a concentration of 100 ng per well onto Nunc MaxiSorp ELISA plates (ThermoFisher) in 50 mM carbonate-bicarbonate coating buffer pH 9.5 (Sigma #C3041) and allowed to bind for one hour at 37 °C. Plates were washed six times with Tris-buffered saline with 0.1% Tween20 (TBS-T), and then blocked with 5% milk in TBS-T for two hours at room temperature. Plates were washed three times in TBST before each antivenom (neat, non-normalised) was diluted 1 in 500 in blocking solution, added to the plate and five-fold serial diluted six times before being incubated overnight at 4 °C. The following day, plates were washed six times in TBS-T and anti-horse or anti-sheep IgG secondary antibodies conjugated to horseradish peroxidase (Sigma #A6917 and #A3415, respectively) were added at 1 in 1000 dilution in PBS for two hours at room temperature. Plates were washed six times with TBS-T and developed with ABTS substrate (0.1 mg/mL 2,2'-azino-bis[3-ethylbenzthiazoline-6-sulfonic acid] diammonium salt [Sigma #A9941] in 0.05 M citrate buffer pH 5.0 with 0.0075% hydrogen peroxide) for 15 minutes at room temperature. The optical density was immediately read at a wavelength of 405 nm (OD_{405}) on a LT-4500 plate reader (Labtech). All measurements were performed in duplicate, and control wells consisting of venom-naïve sheep IgG or horse $F(ab')_2$ (diluted 1 in 25 or 1 in 5 in PBS respectively to match average protein concentration of the antivenoms at 1 in 500 dilution, then serial diluted as per antivenom), as well as secondary antibody only, were also included. Data are available in Supp. File 2.

2.6 Phospholipase A_2 assay

Neutralisation of venom phospholipase A_2 (PLA_2) activity was measured using an EnzCheck Phospholipase A_2 assay kit (Invitrogen, UK #E10217), as previously described [28]. All test conditions were assayed in triplicate for all plates, and plates were measured on a CLARIOStar (BMG Biotech) at excitation 485-15 nm and emission 520-10 nm. For all data analyses, buffer only well values were subtracted from all other values as per manufacturer instructions. A bee venom PLA_2 standard curve (provided in the assay kit) was ran in each assay plate in accordance with manufacturer instructions to determine the specific PLA_2 enzymatic activity, and the specific activity of test conditions were determined by interpolation from the equation of the bee venom PLA_2 standard curve (plotted in Prism 9, GraphPad, RRID:SCR_002798). Optimisation of the amount of venom to be used for each species was first performed to identify the amount of venom that falls within the linear range of enzymatic activity measurements. The relative fluorescence units (RFU) were plotted against the amount of venom per well, and the graphs were manually assessed to identify venom amounts in the linear range of the assay. From these results (shown in Supp. Fig. S1 and Supp. File 3), the optimal venom amounts were determined as 1 µg for *E. romani* (Cameroon), *E. ocellatus* (Ghana) and *E. romani* (Nigeria), 0.5 µg for *E. coloratus*, and 0.25 µg for *E. leucogaster* and *E. p. leakeyi*. RFU was converted to specific PLA_2 enzymatic activity (U/mL/µg) in Excel (Microsoft, RRID:SCR_016137) using the equation derived from the bee venom PLA_2 standard curve to compare the PLA_2 activity of the venoms.

Antivenoms were serial diluted two-fold (in PBS containing the pre-defined amount of venom to 12.5 µL volume per well) in a clear, polystyrene 384-well plate (Greiner BioOne #781101). Final volumes of antivenom in respective wells were 0.10 µL to 6.25 µL. Plates were incubated at 37 °C for 30 minutes then cooled to room temperature, following which 12.5 µL PLA_2 substrate (reconstituted as per manufacturer instructions) was added to each well. Plates were incubated in the dark at room temperature for 10 minutes and then read in a CLARIOstar plate reader (BMG Labtech). RFU measurements were converted to PLA_2 activity using the equation of the standard curve, and then expressed as percentage of activity (where the venom only control was 100% activity) using Microsoft Excel (Microsoft, RRID:SCR_016137). For statistical analyses the data were analysed using two-way ANOVA (multiple comparisons) in Prism 9 (GraphPad, RRID:SCR_002798) to compare the antivenoms at each dilution. For clarity, data shown are the highest four amounts of antivenom, and data for the lower amounts of antivenom are in Supp. File 3.

2.7 Snake venom metalloproteinase assay

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

2.8 Bovine plasma clotting assay

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

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

2.9.1 Ethical approvals

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

2.9.2 Animal maintenance

CD1 mice (male, 18-20 g, Charles River UK) were grouped in cages of five upon arrival (forming the experimental unit) and acclimated for one week before experimentation in specific pathogen-free conditions. No further randomisation was conducted. Holding room conditions were 23°C with 45-65% humidity and 12/12 hour light cycles (350 lux). Mice were housed in Techniplast GM500 cages (floor area 501 cm^2) containing 120 g Lignocell wood fibre bedding (JRS, Germany), Z-nest biodegradable paper-based material for nesting and environmental enrichment (red house, clear polycarbonate tunnel and loft). Mice had *ad lib* access to irradiated PicoLab food (Lab Diet, USA) and reverse osmosis water in an automatic water system. Cages were changed fortnightly with fresh material in the new cage. Cages were selected at random for experimental treatments, and all experiments used mixed gender experimenters who were unblinded to the test articles.

2.9.3 ED_{50} and comparative ED_{100} experiments

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

Table 2. Median Lethal Dose (LD_{50}) and the subsequent 5 x LD_{50} for murine lethality model. Reported LD_{50} values for each of the six *Echis* venoms (with source of reported LD_{50}), and the 5 X LD_{50} dose used for effective $dose_{50}$ (ED_{50}) and ED_{100} experiments in this study.

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

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

The protocols were prepared before the study with the research questions of i) determining ED_{50} and ED_{100} of the three antivenoms against *E. romani* (Nigeria) venom and *E. p. leakeyi* (Kenya) venom and (ii) assessing the survival rates of animals injected with a) *E. romani* (Cameroon) and *E. ocellatus* (Ghana) when given the ED_{100} of antivenom that prevented lethality against mice injected with *E. romani* (Nigeria) venom, and b) *E. coloratus* and *E. leucogaster* when given the ED_{100} of antivenom that prevented lethality against mice injected with *E. p. leakeyi* venom. ED_{50} studies initially used four dose groups (the minimum number to determine an ED_{50} by Probit analysis), and additional dose groups were used if necessary to complete the ED_{50} curve. ED_{50} was determined by Probit analysis using Excel (Microsoft, RRID:SCR_016137).

We used an ED_{100} comparison design for the three additional venoms as opposed to ED_{50}s, which equated to one dose group tested per venom per antivenom, as opposed to at least four dose groups required for ED_{50}. These experiments were designed to provide informative comparative antivenom efficacy readouts with substantially reduced ethical cost via reduction of experimental animal numbers.

<u>2.10 Role of the funding source</u>

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3. Results

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

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

3.2 Antivenom recognition of *Echis* venoms by ELISA

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

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

3.3 Neutralisation of venom PLA_2 activity

The venoms demonstrated considerable differences in enzymatic PLA_2 content, as shown in Fig. 3A ($p < 0.0001$). The venoms of *E. p. leakeyi* and *E. leucogaster* demonstrated highest activity (19.48 and 15.78 [U/mL]/µg respectively). In comparison to *E. p. leakeyi*, the venoms of *E. coloratus* and *E. ocellatus* had 3-fold and 5-fold lower PLA_2 activity respectively, and the lowest activity was observed in the two *E. romani* venoms (0.47 and 1.75 [U/mL]/µg for Nigerian and Cameroonian venoms respectively).

The ability of the three antivenoms to neutralise the PLA_2 activity of these venoms was determined using four different volumes of antivenom (6.25, 3.13, 1.56 and 0.78 µL/well) (Figure 3B-G). All three antivenoms demonstrated strongest neutralisation of PLA_2 activity from Nigerian *E. romani* venom (Figure 3F), with a 95-100% reduction in PLA_2 activity observed at the highest amount of antivenom tested (6.25 µL), and when comparing PLA_2 neutralisation at the lowest volume (0.78 µL), SAIMR Echis showed strongest neutralisation and was significantly more inhibitory than EchiTAbG ($p = 0.001$). The three antivenoms showed comparatively weaker neutralisation of PLA_2 activity from *E. ocellatus* venom and Cameroonian *E. romani* venom (Figure 3D and E). At the lowest volume of antivenom (0.78 µL), no significant differences were detected between the antivenoms against *E. ocellatus* (Ghana) ($p > 0.8$), whereas for *E. romani* (Cameroon) SAIMR Echis was significantly more effective than EchiTAbG and Echiven ($p < 0.015$ for both). All three antivenoms showed comparatively weaker inhibition of the PLA_2 activity of *E. coloratus* venom (Figure 3B), and at the lowest dose of antivenom (0.78 µL) no significant differences between the three antivenoms were observed ($p > 0.99$). The neutralisation of *E. leucogaster* and *E. p. leakeyi* venom PLA_2 activity showed strong differences between the three test antivenoms. At the lowest dose of antivenom, SAIMR Echis antivenom proved the most effective against both venoms ($p < 0.001$ compared to EchiTAbG and Echiven, for both venoms).

3.4 Neutralisation of venom SVMP activity

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

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

3.5 Neutralisation of venom plasma clotting activity

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

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

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

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

3.6 Ability of antivenoms to neutralise murine venom induced lethality

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

Table 3. Antivenom efficacy against *E. romani* (Nigeria) and *E. p. leakeyi* in a murine pre-incubation model. The efficacy of EchiTAbG, SAIMR Echis and Echiven against *E. romani* (Nigeria) and *E. p. leakeyi* reported as ED_{50} (µL), potency (n-1 LD_{50} / ED_{50}) and volume per mg of venom (µL/mg). 95% confidence intervals indicated in parentheses.

***E. romani* (Nigeria)**	**EchiTAbG**	**SAIMR Echis**	**Echiven**
ED_{50} (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)

E. p. leakeyi	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)

We next implemented a comparative dose assay, similar to one previously utilised for comparing the efficacy of various polyvalent antivenoms for East Africa [24], rather than performing full ED_{50}, to reduce the quantity of mice required for comparative testing of each antivenom against each additional venom. Using the ED_{100} dose of each antivenom (the volume of antivenom which conferred 100% protection in experiments to obtain antivenom ED_{50} values) we examined each antivenom's ability to neutralise 5 x LD_{50}s of *E. romani* (Cameroon) and *E. ocellatus* venoms (using ED_{100}s determined against Nigerian *E. romani*) or *E. leucogaster* and *E. coloratus* venoms (using ED_{100}s determined against *E. p. leakeyi*). Of note, the reported ED_{100} volume for EchiTAbG only conferred 80% protection (100% protection was not achieved in the ED_{50} experiment).

Table 4. Antivenom volume to confer 100% survival (ED_{100}) against *E. romani* (Nigeria) and *E. p. leakeyi* in a murine pre-incubation model. The ED_{100} (volume of antivenom that conferred 100% survival) of the test antivenoms EchiTAbG, SAIMR Echis and Echiven against *E. romani* (Nigeria) and *E. p. leakeyi* in the pre-incubation model of envenoming. All assays used a venom challenge dose of 5 x LD_{50} (Table 2).

Venom	EchiTAbG	SAIMR Echis	Echiven
E. romani (Nigeria)	100 µL	25 µL	30 µL
E. p. leakeyi	90 µL	50 µL	60 µL

When using the ED_{100} doses (ED_{100} dose observed for *E. romani* [Nigeria], Table 4) of each antivenom against 5 x LD_{50} of *E. romani* (Cameroon), SAIMR Echis and Echiven provided partial protection at the end of experiment (80% survival and 40% survival, respectively and mean survival times 314 minutes and 201 minutes, respectively) (Figure 7C and 7E), while the mean survival time for EchiTAbG was 99 minutes (20% survival). When using the same ED_{100} doses against 5 x LD_{50} of *E. ocellatus* (Ghana), both SAIMR Echis and Echiven provided complete protection, with 100% of mice surviving until the end of experiment. In contrast, EchiTAbG failed to prevent lethality but further increased time to humane endpoint (mean survival times 95 minutes, compared to 6 minutes with no antivenom). Challenging mice with 5 x LD_{50} of *E. coloratus* venom preincubated with the ED_{100} dose for each antivenom (the ED_{100} dose observed for *E. p. leakeyi*, Table 4) resulted in 0% survival with EchiTAbG and Echiven, and

20% survival with SAIMR Echis (Figure 7A). In contrast, using the same ED_{100} doses, SAIMR Echis and Echiven performed well against 5 x LD_{50} of *E. leucogaster* venom (conferring 100% and 80% survival respectively at 6 hours), whilst EchiTAbG failed to confer full protection against venom-induced lethality but increased mean survival times to 124.8 minutes from 7.8 minutes without antivenom (Figure 7B and 7E).

Discussion

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

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

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

The main proteinaceous components of *Echis* venoms are SVMPs, PLA_2, C-type lectin-like proteins, serine proteases, disintegrins, and L-amino acid oxidases [17,21,39]. In particular, the *Echis* genus of snakes have a remarkably high abundance of SVMPs but with substantial variations in quantity observed between individual species; SVMPs comprise up to 70% of *E. ocellatus/romani* venom, whilst *E. leucogaster* contains only 27% [40], and this variation was reflected in *in vitro* SVMP activity. These zinc dependent proteinases play a fundamental role in driving life-threatening venom-induced consumption coagulopathy and systemic haemorrhage [41]. The *in vitro* SVMP activity of each of the six venoms were significantly neutralised by the highest dose of each antivenom, however, it must be noted that SAIMR Echis and EchiTAbG performed poorest against *E. p. leakeyi* venom in comparison to the other venoms. The failure or poor *in vitro* neutralisation of the SVMP activity of *E. p. leakeyi* at the highest dose tested may reflect the relatively high abundance of SVMPs belonging to the PII family in *E. p. leakeyi* venom compared to the low abundance of PII SVMPs in the venoms of *E. ocellatus/romani* against which the antivenoms were raised. A previous transcriptome analysis of various *Echis* species [42] demonstrated proportional differences in relative Group II PLA_2 abundance, with *E. p. leakeyi* containing the highest expression of PLA_2 transcripts and markedly less detected in *E. romani* (previously *E. ocellatus*) and *E. coloratus* [42]. Similarly, venomic analyses of *E. romani* (previously *E. ocellatus*) from different locales demonstrated intraspecies differences in the abundance of PLA_2 [43], and this was demonstrated by evident differences observed in our *in vitro* PLA_2 assay. Bearing in mind the known variation in venom toxins and subsequent activity, this reiterates the importance of carefully evaluating the source of venoms used for antivenom production and the need for thorough and transparent preclinical testing of proposed species efficacy.

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

have implications for their clinical use. The calculated ED_{50} values were in broad agreement to previously calculated ED_{50} values for EchiTAbG and SAIMR Echis against Nigerian *E. romani* (formerly *E. ocellatus*) venom and SAIMR Echis for *E. p. leakeyi* venom [22], with SAIMR Echis possessing the most potent venom-neutralising ability (lowest ED_{50} and highest potency value) against both *E. romani* (Nigeria) and *E. p. leakeyi*. In comparison, Echiven was two-fold less potent against both venoms and EchiTAbG was 7-fold and 13-fold less potent against the venoms, respectively. When accounting for total protein concentration of the three *Echis* monospecific antivenoms (Table 1), the differences in neutralising efficacy between products were more modest, although the trends in neutralising ability remained.

When the antivenoms were assessed further for cross-reactivity, intra-country and intra-species differences became apparent. The dose of each antivenom that neutralised 100% of lethality against *E. romani* (Nigeria) was unable to fully protect mice challenged with *E. romani* venom from Cameroon, whilst SAIMR Echis and Echiven fully prevented lethality from *E. ocellatus* but EchiTAbG failed to prevent lethality from this venom. We believe these differences in efficacy could be explained by different challenge doses used for the different venoms, but further investigation such as full ED_{50} experiments would be useful to identify whether the antivenoms can provide full protection. Similarly, SAIMR Echis and Echiven showed strong cross-reactivity with *E. leucogaster*, whilst EchiTAbG failed to prevent lethality from this venom. In contrast to previous studies that demonstrated EchiTAbG could neutralise the lethal effects of *E. coloratus* [24], in our study all three antivenoms were ineffective against this venom. A larger volume of EchiTAbG was used than the previous study, and the same challenge dose of venom was used in both, so these findings were unexpected and could potentially be due to batch variation of either the challenge venom or the antivenom. Although the three antivenoms in this study lacked efficacy against Egyptian *E. coloratus* venom, it would be worth confirming that the commercial *E. coloratus* antivenom produced in Israel [44,45] has good efficacy against this venom to ensure adequate treatments against bites from this species.

To uphold principles of the 3Rs (replacement, refinement and reduction of animals in research), we used a comparator model of envenoming [24] which enables prediction of antivenom performance but requires fewer mice than full ED_{50} testing. We challenged mice with either the venom of *E. romani* (Cameroon) and *E. ocellatus*, or *E. leucogaster* and *E. coloratus*, with corresponding antivenom doses which provided 100% protection against *E. romani* (Nigeria) and *E. p. leakeyi*, respectively. The results of the ED_{100} comparator assays mirrored the *in vitro* and ED_{50} findings, with substantial differences in dose-matched potency of antivenoms against other species. The most notable and perhaps

unexpected finding was the reduced or poor ability of antivenoms to protect mice from *E. romani* (Cameroon) envenoming when using the ED_{100} *E. romani* (Nigeria) antivenom dose, with 60% and 80% of mice succumbing to venom effects when dosed with Echiven and EchiTAbG, respectively. This suggests that *E. romani* venoms from different localities have different potencies and thus differences in their ability to be neutralised by antivenoms, meriting further research to understand the impact of intraspecific *E. romani* venom variation on antivenom efficacy. This illustrates how, given the frequency of sometimes extreme venom variation within species, even in the face of extensive gene flow [46], taxonomic revisions should be seen as broad roadmaps for additional research into antivenom efficacy, but not interpreted as robust predictors of venom composition or antivenom effectiveness [47]. In view of the public health importance of the *E. ocellatus* complex, further research into variation in venom composition within the group would be advisable. It is also important to keep in context here that only a single dose has been examined to enable comparative analysis of an antivenom's ability to neutralise different venoms at that dose. The failure of an antivenom to protect mice from envenoming in these experiments, while it is indicative of potential potency of an antivenom, it is not capable of definitively saying if an antivenom is ineffective, and results should therefore be viewed in this context and treated with caution.

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

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

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

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Declaration of Interests

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

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Data sharing

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

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Figure Legends

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

Figure 2. The titre of three antivenoms against six *Echis* venoms determined by end-point titration ELISA. EchiTAbG shown in magenta, SAIMR Echis shown in teal, Echiven shown in blue. Venom-naïve equine $F(ab)'_2$ shown in black and venom-naïve ovine IgG shown in purple. Panel a: *E. coloratus*. Panel b: *E. leucogaster*. Panel c: *E. ocellatus* (Ghana). Panel d: *E. romani* (Cameroon). Panel e: *E. romani* (Nigeria). Panel f: *E. p. leakeyi*. Data points represent the mean of two replicates and error bars show the standard deviation.

Figure 3. PLA_2 activity of six *Echis* venoms and their neutralisation by the three different antivenoms. a: PLA_2 activity of *E. romani* (CAM = Cameroon and NGA = Nigeria), *E. ocellatus* (GHA = Ghana), *E. coloratus*, *E. leucogaster* and *E. p. leakeyi*. Samples were subtracted for background and converted to activity in (U/mL)/µg by extrapolation from a bee venom standard curve. Data show the mean of three replicates and error bars represent standard deviation. Statistical differences in activity compared to 'buffer only' were determined by one-way ANOVA, with venom specific p values indicated above the bar (ns = not significant, $p > 0.05$).
b-g: Neutralisation of (b) *E. coloratus*, (c) *E. leucogaster*, (d) *E. ocellatus* (Ghana), (e) *E. romani* (Cameroon), (f) *E. romani* (Nigeria), (g) *E. p. leakeyi* PLA_2 activity by the three antivenoms EchiTAbG (ETG), SAIMR Echis (SE) and Echiven (EV) at different doses, expressed as a percentage of a no antivenom control showing 100% activity. Data show the mean of three replicates and error bars represent standard deviation. Two-way ANOVA was performed to compare differences in PLA_2 activity at the 0.78 µL dose of antivenoms. * indicates $p < 0.05$, **** indicates $p < 0.001$, ns = not significant ($p > 0.05$).

Figure 4. SVMP activity of six *Echis* venoms and their neutralisation by the three different antivenoms.
a: SVMP activity of *E. romani* (CAM = Cameroon and NGA = Nigeria), *E. ocellatus* (GHA = Ghana), *E. coloratus*, *E. leucogaster* and *E. p. leakeyi*. Data show the mean of four replicates and error bars represent standard deviation. Statistical differences in activity compared to PBS, and between

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

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

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

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

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

Figure 6. ED_{50} of the three antivenoms against *E. romani* (Nigeria) and *E. p. leakeyi* against 5 x LD_{50} venom dose.

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

reported in volume (µL of antivenom). b: Potency, where potency is calculated as (n-1 LD_{50})/ED_{50}. c: ED_{50} reported in µL of antivenom per mg of venom.

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

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

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

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