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RESEARCH ARTICLE

Digital Potency Measurement: A New Approach Methodology for Antisera Effective Dose Assessments

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ABSTRACT

Polyvalent antivenom potency tested for every batch and at four different stages of production is a regulatory requirement to be followed by the industry. Antivenom manufacturers have been following the gold standard test methodology for estimating median effective dose in mice for ages. Here, we report a non-animal New Approach Methodology that aligns with 3Rs in animal testing agenda, leveraging in vitro human stem cell technology for recreating microphysiological system complemented with process automation, Artificial Intelligence and Machine Learning digital workers' effective utilization in the assay system to measure *Naja naja* snake antivenom potency. In vitro neutralization performed on specially configured human Microphysiological System acquired phenotype data sets at 20X magnification were analyzed against benchmark panels in the trained prediction model while the Artificial Intelligence predicted median effective dose value of venom-antivenom mixture was 3.9µL, showing a potency value of 2.04 mg recorded as the readout. This method adopted in the antivenom producer's workflow will reduce reliance on mice-based testing and showcases potential for acceptance of robust alternative strategy to traditionally practiced protocols.

Keywords: Artificial Intelligence, human stem cells, venom, antivenom, IC_{50}, potency

Introduction

Snake bite is a serious public health issue in tropical and subtropical countries. Envenoming affects all ages of victims causing pathophysiological effects in the human body leading to death. Polyvalent antivenom raised against the venom from "Big-four snakes of India"- Spectacled Cobra, Common Krait, Russell's viper and Saw-scaled viper is used as therapy for snakebite envenomation. As per the WHO guidelines, antivenom manufacturers must rely on stringent quality check points, parameters before releasing the product batches into the market. Antivenom potency is considered to be the most critical parameter that defines the effective dose of the product and mandated to be tested at various stages of its production [1-3]. It is defined as the amount of venom (in mg) neutralized per ml of antivenom. The gold standard method of measuring the potency of antivenom is by calculating median effective dose (ED_{50}) that requires up to 300 mice per batch for testing [4]. This routine quality testing step not only causes cruelty to the animals but also adds cost to the production as manufacturers must maintain animal facilities which in turn should comply with CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) guidelines [5]. These are not experimental animals at the preclinical stage in developing a new intervention for human consumption but are animals bred to be sacrificed day in and day out of the antivenom industry's universe. The same WHO along with Committee on Biological Standardizations acknowledges that animal tests have physiological limitations since venom/antivenom injection protocols do not represent the natural situation, and the physiological responses of rodents to envenoming and treatment may differ from those of humans [6]. These two agencies as part of the guidelines given in 2016 on production, control and regulation of snake antivenom immunoglobulins have recommended that all practitioners of animal testing should prioritize implementation of 3Rs into these tests to reduce the substantial number of mice used, and their collective pain, harm and distress [7,8].

Therefore, there has always been a compelling need to implement 3Rs in the use of mice for routine ED_{50} measurements of antisera products produced in batches; while in recent years different strategies involving *in vitro* assays for testing antivenom potency has gained momentum in the field of toxicology [9,10] . In vitro measurements such as Phospholipase A2 - PLA2, cytotoxicity and procoagulant effects of *Bothrops* venom were correlated with the in-vivo lethality assay [11]. On the other hand, antivenom efficacy tested using Enzyme-Linked Immunosorbent Assay-ELISA has shown poor correlation with *in vivo* ED_{50} values and has been inconclusive[12,13]. In vitro assays are categorized into three types: (1) Binding assays that measure the ability of antivenom to form complexes with the venom toxins, such as ELISA[12,14] and chromatography techniques [13,15]; (2) Functional assays that assess neutralization potential of antivenom against specific venom effects, such as coagulation, Phospholipase A2 (PLA2) toxicity, Proteolytic activity, L-amino acid oxidase toxicity, Hyaluronidase toxicity [16-18]; (3) Cellular assays that measure the extent of cytotoxicity/neurotoxicity by venoms and its prevention by antivenom [19].

We have developed a New Approach Methodology (NAM) as an in vitro assay system leveraging stem cell technology (human Microphysiological System - hMPS) and process automation on Artificial Intelligence - AI and Machine Learning - ML digital workers to measure antivenom neutralization capacities (Figure 1). Here, we present its use case of digital potency measurement against *Naja naja* venom in the assay system which requires no extrapolations. The assay system has two components: 1. A well characterized hMPS [20] 2. A trained software that picks phenotype signals from treated component 1 (in vitro neutralization on hMPS) to publish potency as read out. We propose this NAM as a cruelty free assay system aligning with 3Rs agenda of the practitioners, and can be seamlessly integrated in the antivenom production workflow to report rapid ED_{50}, potency like scores in the most humane manner as part of routine testing.

Figure 1

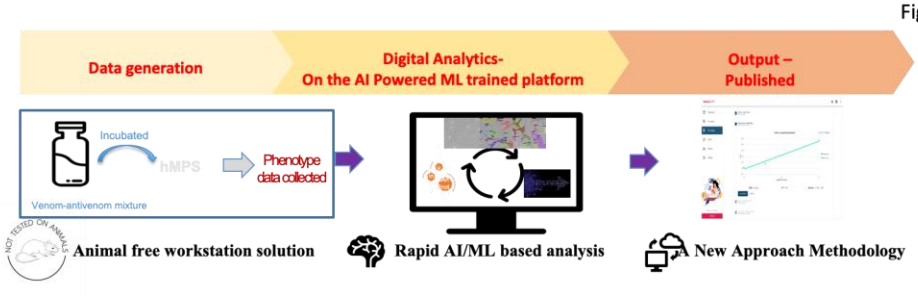


Figure 1: Schematic of the New Approach Methodology for publishing the potency value of the antivenoms.

Material & Methods:

Biomolecules used in the assay:

Naja naja (cobra) venom (10mg/ml), antivenom (lyophilized powder with potency of 0.6mg/ml, on mice measured as 1ml neutralizing 0.6mg of cobra venom) and other venoms were received from the collaborator's lab (VINS Bioproducts Ltd, India) and were handled in compliant with biosafety protocols. Bungarotoxin is from Sigma (203980).

Human micro physiological system - hMPS:

A primary, processed stem cell composed special hMPS characterized previously [20] is the seed harvest. The hMPS is configured to constitute progenitors of neuro and hemato cellular moieties in ratio, supplemented with dynamic microenvironment of culture conditions as in vitro platforms in units in this protocol.

Trained Software presenting features:

Back bone	Residual neural network - ResNET101
Network	Convolutional Neural Networks - CNN
Model Version	Version 101
Maximum detections per instance	100
Training AI	Supervised training on Neural Networks
Features	Modular design allows analysis of single cells and new phenotypes. Flexible to add new modules, specific to ONLY hMPS cell type
Technical description for the tool	The platform is available as a progressive web application for the end-consumer, which facilitates rapid testing and analysis. It is available at https://neurosafe.transtoxbio.com/login
Input data qualification	2D images taken under 20X magnification under phase contrast microscope; Gene expression data values; Numerical values
Image analysis	Performed on concept of pipeline of individual modules while each module processes the image in sequential order: object identification followed by measurement
Annotations	Healthy cells, Cells in shock, Dead cells, Apoptotic cells, Necrotic cells
Cells identification	Identification based on cells shape, speckles in the cells, nucleus shape, cell aggregation
Plotting data tools	Uses probit analysis, log values, excel to draw straight line graph
Source code	Closed source system with a proprietary license model

Venom treatment on human MicroPhysiological System

Cells were seeded in 6 well-plate at a density of 20,000 per well and incubated overnight (12hr) for adherence on the surface of the well. The spent medium from these wells was replaced with the medium containing increasing concentrations of venom and incubated for 5 hours (Figure 2A). After the incubation period, the cells were imaged under 20X magnification in an inverted phase contrast microscope. Up to 30 images were captured per well and stored in a folder with labels in the attached computer system until uploaded into the software for measuring IC_{50}.

In vitro Neutralization on human MicroPhysiological System

10 µg of venom (5*IC_{50} considered as challenge dose) was preincubated with different amounts of antivenom at 37 C for 30 min. The mixture was added to the cells in 6 well-plates and the plates were incubated at 37 C with 5% CO2 for 5 hours. The plates were removed from the incubator and the cells were imaged under the phase contrast microscope at 20X magnification (Figure 2B).

Statistics & Prediction Model:

Coefficient of variation (CoV) for each data set was calculated as the ratio of standard deviation to the mean and the value was shown as the extent of variability in relation to the mean of the population. The higher the value of CoV, the greater the dispersion; hence the cut off value kept was less than 1 as standard in the assay system. IC_{50} value of venom was measured by constructing a dose response graph plotted between log value of concentration vs probit values of affected cells' percentages. Transformation concentration data to logarithm (log10 or loge) scale was taken for the linear regression curve [21]. The acceptance criteria of the linear regression in the assay (R2) were kept as ≥ 0.70. The standard error of mean as the ratio of standard deviation to the root of sample size. The effective dose (ED_{50}) to prevent 50% cell death was calculated for each test using Miller and Tainter method by plotting a straight-line graph between log volume vs probit percentages values. The

potency of the antivenom sample was calculated by estimating the ability of the antivenom to protect the cells against the lethal effect of the venom. It is usually expressed as 1ml of polyvalent antivenom that can neutralize particular concentration of venom. The formula used for calculating the antivenom potency against venom in the assay was IC_{50} (µg)/ED_{50} (ml) X (n-1)/1000 = Potency (mg/ml); where n is the number of times the test dose of the venom considered as challenge dose in the neutralization assay [3].

Results:

The AI could publish venom IC_{50} value measured from the images as AI.IC_{50} (Artificial Intelligence. Inhibitory Concentration)**:**

The toxicity evaluation of the venom as IC_{50} was a critical step in the assay system set out to measure antivenom potency. The software was trained to pick the signals of Affected Cells (AC) from the images and could publish IC_{50} patterns consistent to venom concentration as cytotoxicity on cells. As shown in Figure 2, the prediction model was able to pick the percentage of AC proportional to the toxicity of the venom added while the half maximal inhibitory concentration was determined by the dose of venom that showed 50% of the AC [21] (Table 1). The percentage of AC were quantified by the AI tool embedded in the trained software which was transformed to probit values (Table 1&2).

Table 1: Generation of Machine derived percentage of Affected Cells from the phenotype data per concentration of venom

0.5µg*								
File Name	Healthy Cell	Dead Cell	Cell in Shock	Necrotic Dying Cell	Apoptotic Dying Cell	Nucleus		
0011.png	9	1	3	0	0	9		
0024.png	8	1	2	0	0	7		
0014.png	1	1	4	0	0	3	% AC	34.09
0013.png	8	0	8	0	0	6	Mean	5
0015.png	15	1	4	0	0	12	SD	1.788854
0025.png	10	0	5	0	0	7	CoV	0.357771
0021.png	15	1	6	0	0	11		
0022.png	5	0	2	0	0	4		
0012.png	17	1	6	0	0	12		
0023.png	9	2	2	0	0	9		

*Shown is the value for 0.5µg *Naja naja* venom treatment with 10 images.

Table 2: Results of percentage Affected Cells with increasing doses of venom for determination of IC_{50}.

Venom (µg)*	**%AC****	**Log of Venom concentration**	**%AC (Probit value)*****
0.5	34.09	-0.30103	4.59
1.5	47.41	0.1760913	4.9
3	48.32	0.4771213	4.95
5	51.16	0.69897	5.03
10	81.36	1	5.88
20	89.36	1.30103	6.23

*Concentration of Naja naja venom used for treatment on hMPS.
**Represented is the median value for triplicate readings.
*** Transformation of percentage Affected Cells to probit values.

These probit values were plotted against log doses of the venom concentrations and the venom concentration corresponding to the probit 5 was considered as the IC_{50} value (Figure 3A). The mean AI predicted IC_{50} value published for cobra venom was 2.8 +/- 0.83 µg in the assay system.

Figure 2

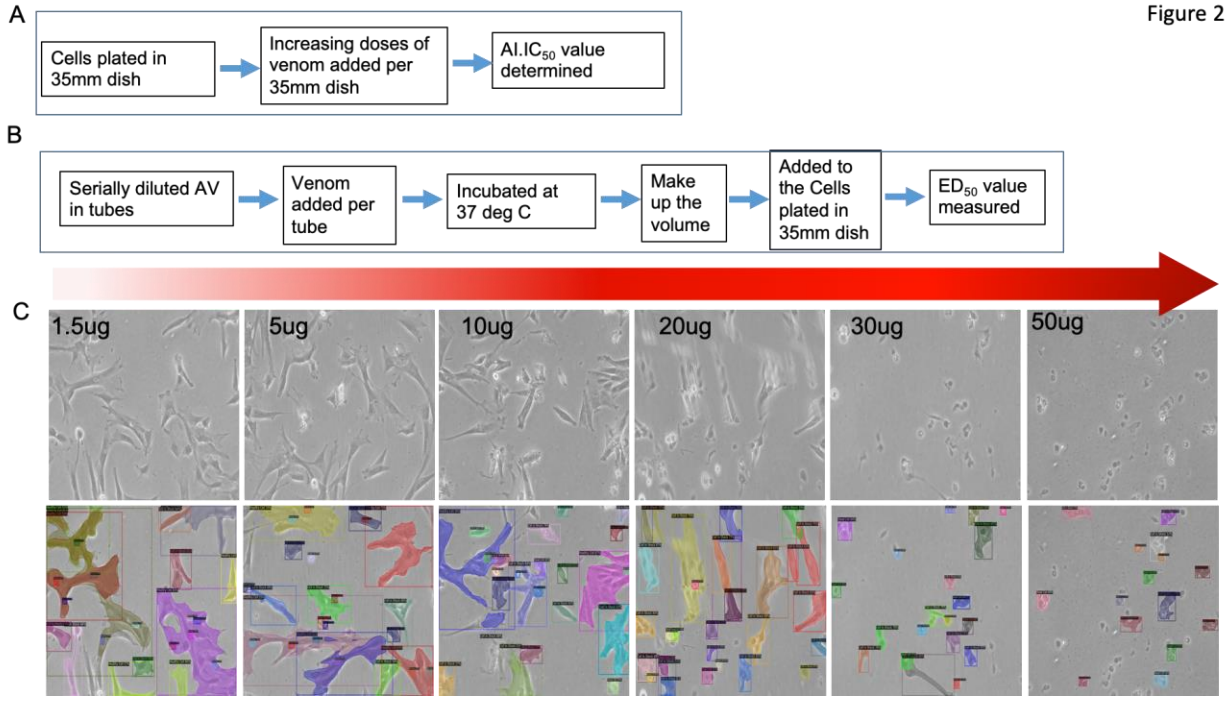


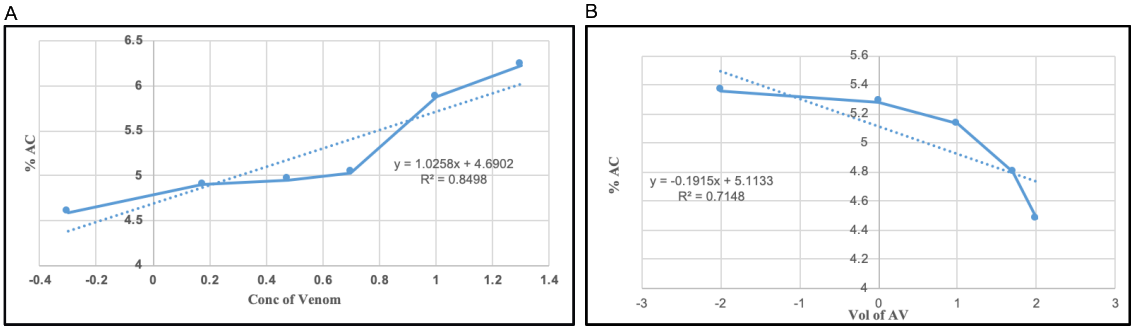
Figure 2- Digital Potency Assessment: (A). Scheme followed to measure IC_{50} value of the venom. (B). Schematic for the measurement of ED_{50} of the antivenom. (C). Artificial Intelligence Prediction: Median IC_{50} value determination - the cells were treated with increasing concentrations of venom and phase contrast microscopic images were captured at 20X magnification as shown in the 1st row. The corresponding Artificial Intelligence augmented images for prediction from the affected cells were depicted in the 2nd row.

In vitro Neutralization on human MicroPhysiological System generated phenotype as input data in the prediction model:

Traditional venom neutralization assays include a step for incubation of venom with antivenom aliquots to allow antibody binding prior to their exposure to the cell model or test animal. In the *in vitro* NAM as shown in Figure 1B, 10µg of venom (~5*IC_{50} considered as challenge dose) was preincubated with different amounts of antivenom at 37^{0}C for 30 min. The mixture was added to the cells in 6 well-plates and the plates were incubated at 37^{0}C in an atmosphere of 5% CO_2 for 5 hours [11]. The plates were removed from the incubator and the cells were imaged using a phase contrast microscope at 20X magnification. The hMPS was also treated with antivenom and naïve IgG (Sigma, I 4506) and the corresponding phase contrast microscopy-based images at 20X magnification served as negative controls. In this method, the venom-antivenom mixture (Antiserum I.P. ViNS Bioproducts Ltd, B.No: 01AS22038; MFG: 03/2022; EXP: 02/2026) incubated hMPS held the biological information on the neutralization. The AI quantified the percentage of affected cells from the micro physiological system exposed to venom-antivenom mixtures led to ED_{50} calculations in the software (Table 3). The ED_{50} value of antivenom against *Naja naja* venom measured was 3.9µL (Figure 3) while the potency value published was 2.04mg, which is not significantly different from the mouse-derived potency value (p=0.157, statistical significance was determined using Student's two-tailed *t*-test). In this method, the venom-antivenom mixture incubated cells held the biological information on the neutralization.

Figure 3

Figure 3



Deriving the Scores. (A) Analysis was done on 10 images per treatment is shown for the concentration 0.5µg. CoV in the table stands for coefficient of variation for each data set and was calculated as the ratio of standard deviation to the mean and the value was shown as the extent of variability in relation to the mean of the population. The higher the value of CoV, the greater the dispersion; hence the cut-off value kept was less than 1 as standard in the assay system. IC_{50} value of venom was measured by constructing a dose-response graph plotted between log value of concentration vs probit values of affected cells' percentages. Transformation concentration data to a logarithmic (log10 or loge) scale was taken for the linear regression curve[21]. The acceptance criteria of the linear regression in the assay (R2) were kept as ≥ 0.70. The standard error of the mean is the ratio of the standard deviation to the root of sample size. A straight line graph was generated by plotting the points for concentration in log values as x-axis and percentage of affected cells as probit values in y-axis. The derived straight-line equation is 1.0258x+4.6902. AI-derived IC_{50} value is 2.004µg. (B) Represents the generation of potency value for the antivenom. Consolidated data table (Table 3) was generated for a percentage of affected cells for each volume of antivenom used. The effective dose (ED_{50}) to prevent 50% cell death was calculated for each test using Miller and Tainter method by plotting a straight-line graph between log volume vs probit percentages values. The potency of the antivenom sample was calculated by estimating the ability of the antivenom to protect the cells against the lethal effect of the venom. It is usually expressed as 1ml of polyvalent antivenom that can neutralize a particular concentration of venom. The formula used for calculating the antivenom potency against venom in the assay was IC_{50} (µg)/ED_{50} (ml) X (n-1)/1000 = Potency (mg/ml)[22]; where *n* is the number of times the test dose of the venom considered as challenge dose in the neutralization assay. A straight line graph was generated for the log values of antivenom volume on the x-axis with probit values of percentage affected cells on y-axis. Median effective dose of antivenom, ED_{50} was calculated based on the generated straight-line equation: -0.1915x+5.1133. The AI-ED_{50} value determined is 3.9µl. Potency is calculated as (n-1) multiplied by of the ratio of IC_{50} over ED_{50} where n determines the challenge dose given to the cells; anti-venom potency for *Naja naja* venom is (4X2.00/3.9) 2.046mg.

Table 3: Generation of percentage affected cells with treatment of the neutralization mixture to determine the ED_{50}

Vol of AV(µl)*	%AC**	Vol in µl(log)***	%AC (Probit value)****
0.01	64.04	-2	5.36
1	61.5	0	5.28
10	55.6	1	5.13
50	42.08	1.69897	4.8
100	30.42	2	4.48

*Volume of Antivenom used for each neutralization with 5 times of venom.
**Represented is the median value for triplicate readings.
***Logarithmic value for the volume taken in microliters.
****Transformation of percentage affected cells to probit values.

Discussion

Here, we report the application of an AI/ML based prediction model embedded in New Approach Methodology as an assay system for analyzing the cytotoxicity induced by the *Naja naja* venom and the corresponding in vitro neutralization on hMPS.

The strength of this assay system **(Figure 1)** depends on the effective detection (accuracy of prediction by the trained software) of the affected cells (AC) against healthy cells while the type of hMPS utilized is an in vitro surrogate to human physiology mimicking human bodily responses while being alternative to animal system. This special in vitro platform is the choice as the configured system composed of cellular moieties with gene expression profiles representing both human neuro and hematopoietic systems that are the first line of affected architecture by the snake bite. Additionally, this real time in vitro platform has been shown to be phenotypically responsive to the slightest induction with toxins, that can be imaged and is equated to the corresponding genotype barcodes [23]. The prediction model is solely dependent on the ML aspect of the digital framework with supervised wet lab data generated on in vitro system. The plain images of treated (either venom and venom-antivenom mixtures) in vitro system obtained under the phase contrast microscope were used as training data sets in developing the assay system.

The training data sets were generated by using relevant positive controls (cells treated with four different venoms of five different concentrations each at 3 different time points and *in vitro* neutralizations with antivenom batch dated July 2022 from the collaborator's laboratory) and negative control (cells treated with their growth medium without venoms or venom-antivenom mixture) sets, totalling 10,000 data points. These micrographs generated with known concentrations of venoms and antivenoms were trained on a CNN based RESNET 101 architecture [24] using supervised training models on convolutional neural networks (CNN). The software features include modular design allowing analysis of single cells and new phenotypes, flexibility in adding new modules. We annotated each feature from the images and assigned all the tracked labels to one of the categories: i) healthy cells ii) cells in shock iii) nucleus iv) dead cells v) necrotic cells and vi) apoptotic cells. Identification of each category is based on the shape of cells, speckles, granulation in the cells, cell aggregation, shape of the nucleus like phenotypes. Image analysis was performed on the concept of pipeline of individual modules while each module processes the image in sequential order. The large sets of phenotype perturbations of in vitro system created for training the machine became the benchmark panels against which any similar signal picked by the machine supports the quantification of affected cells, which is the basis of the prediction model. The same configuration was treated with various neurotoxins, hematotoxins with sub optimal concentrations [23] to elicit various morphological patterns by our group and speculated the concept of building authenticated patterns as benchmark panels.

New Approach Methodology in the assay system is different from the other cell-based assays

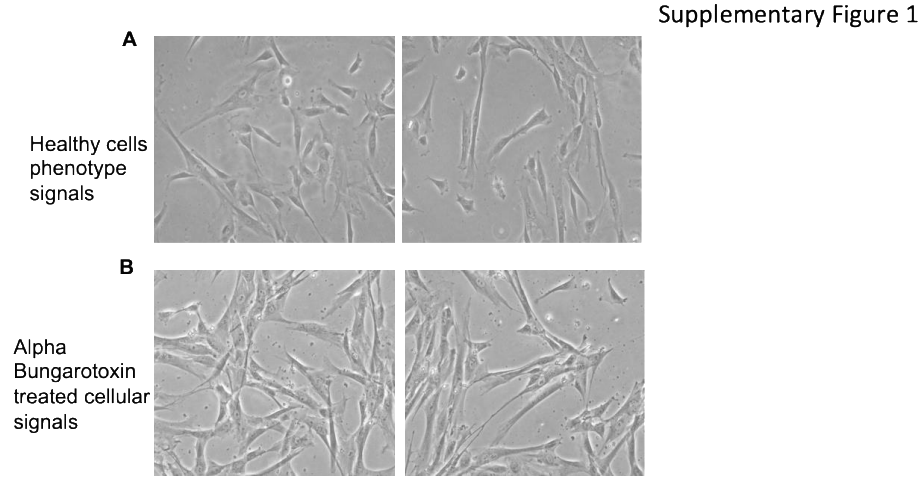
The venom LD_{50} measurement is the prerequisite value for neutralization assay in testing the efficacy of antivenoms and is based on administering different doses in mice followed by recording the percentage of dead animals [4]. Despite being efficient in testing, the procedure is extremely laborious, expensive and time consuming [11,25]. In very few cases neutralization assay is not possible as envenoming is characterized by intense local tissue damage [26]. Considering these issues, alternative in vitro approaches have been in development for estimating the efficacy of antivenoms. These in vitro methods explored measure either binding affinities of antivenom towards venom or PLA2 activity or cytotoxic activity of the venom on a particular cell system; however, use transformed cell lines of human and mouse origin that are known to show poor correlation for human consumption [27]. The routinely practiced cytotoxicity assay measures the number of viable cells or dead cells from the cell culture model treated with the toxin or drug compound while cell viability assays that are currently available use a known toxin as a positive control to measure the marker [28,29]. On the other hand, there are a few publications on AI in the field of cell biology for understating various key aspects like differentiation, identification of cells, identification of subcellular organelles etc. For example - Hay and Parthasarathy used AI to identify bacteria from a 3D microscope images of larval zebrafish gut with 90% accuracy [30] ; Eulenberg in 2017 identified the cell cycle phases in Jurkat cells with high accuracy [31], the application CellProfiler to detect and analyze cells etc [32]. Most of these studies have supported in understanding the cellular mechanisms on stained cell-based platforms followed by imaging while the staining, labeling steps can cause unwanted perturbations induced at the cellular level which may interfere in the interpretation of the results.

In contrast, the reported NAM uses a hMPS model composed of live progenitor, primary cells and the software that is made intelligent with sets of positive control exhibited signals on the same in vitro model as layers of benchmark panels bringing the digital flavor along with robustness, precision in the assay system. Our work took inspiration from Kusumoto Dai, 2018 who used AI deep learning to classify

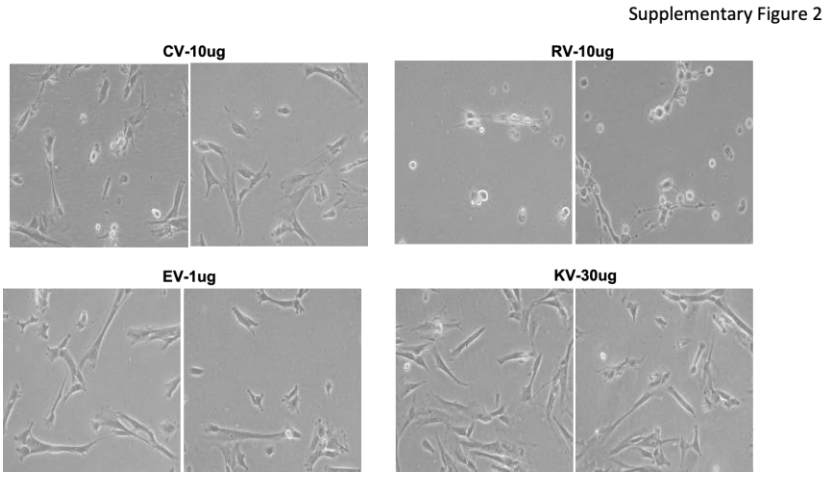
iPSC (induced pluripotent stem cells) vs iPSC derived endothelial cells based on the morphology without the need for immunostaining or lineage tracing [33]. Also, in another study, CeCILE- a deep learning algorithm was used to detect and analyze the cell's reaction to radiation using videos obtained from phase contrast microscopy [34]. Dongyoung Kim, 2019 has used transmitted light microscopy for subcellular structure identification with 99% accuracy based on developed 3 functional AI based models - CellNet, ClassNet, and TrackNet. These networks use fixation of cells to capture the images and to detect the changes [35].

It is known that the venom from the cobra snake that belongs to Elapidae family induces neurotoxic (through nicotine acetyl choline receptors) and cytotoxic activity [36,37] The NAM being discussed here with ML tool has trained the software with neuro and cytotoxic phenotype patterns acquired from treated in vitro platform as part of the training the model exercise. The embedded AI picks signals with confidence against the control patterns to detect toxicity-induced cellular phenotypes (Supplementary Figure 1&2) in the assay system while the derived IC_{50} and potency values reflect the measurable score. The data science, which is a quintessential element in this NAM theorizes that the higher the number of training data sets annotated in the model acquired from in vitro platform treated with venoms and antivenoms collected from multiple authentic sources are, the higher the accuracy rate will be of the assay system's user-friendly prediction model proposed to fit into the existing workflows. The NAM developed as an assay system is robust with concurrent measurements, concentrations tested, number of replicates at each concentration, and response values in consensus with prediction model parameters.

Supplementary Figure 1
Cellular morphologies of the in vitro platform acquired upon treatment with Bungarotoxin which is a known neurotoxin.



Supplementary figure 2
Various cellular morphologies of the in vitro platform acquired upon treatment with venoms.



Studies on the efficacy of antivenoms highlighted and debated discrepancies attributed to the heterogenous sources of the venoms, the lack of standardization in the antivenom production process and the lack of clinical output data points [17,38]. We believe that one such effective manner to control the discrepancies with data and surrounding the potency measurements could be the automation of the assay system qualifying the product for consumption. Additionally, training the assay system with the antivenom producer's specific venoms sourced from identified suppliers; along with embedding the phenotype patterns of in vitro neutralizations in the hMPS (in the place of an animal) as the second set of benchmark signals while implementing the assay system can mitigate the variations of the data. The technology employed in this report predicted (with >92% confidence) the efficacy of antivenom inhibiting the cyto and neurotoxic activity of *Naja naja* venom effectively by integrating AI digital workers. Furthermore, it may enable and make possible the development of widely paraspecific antivenoms which require the use of many mice for testing efficacy. As shown in this report for *Naja naja,* the same NAM published potency values for other snake antivenoms (Supplementary table 1) demonstrates the relevance and the range of the prediction model developed as parallel assay system for mice-based measurements.

Supplementary Table 1:
Comparison of data generated on NAM vs *in vivo* mouse data
CV: Cobra Venom; KV: Krait Venom; Russell's venom; EV: Echis Venom
AV: Antivenom

	Mouse data		**NAM data**	
	LD_{50} (µg)	**Potency (mg/ml)**	**IC_{50} (µg)**	**Potency (mg/ml)**
Naja naja (CV)	11.75	-	2.81	-
Bungarus caeruleus (KV)	2	-	5.7	-
Daboia russelii (RV)	8	-	0.505	-
Echis carinatus (EV)	12	-	0.08	-
CV-AV	-	0.73	-	2.09
KV-AV	-	0.45	-	1.15943
RV-AV	-	0.6	-	Insufficient data
EV-AV	-	0.45	-	1.02591

Conclusion

Here, we reveal a novel use case of digital potency measurement or venom neutralizing capacity of antivenom assessment on a suitable *in vitro* platform as a non-animal NAM. We have incorporated an artificial intelligence (AI) process automation that minimizes inconsistencies in routine assay performance to measure potency values. Hence, we propose a seamless integration of this assay system into an antivenom producer's workflow that produces the finished product through three precursor stages: plasma, clarified bulk, formulated bulk. While measuring potency at all stages is a must as per the WHO guidelines, our NAM is an attempt to support the 3Rs objective to be practiced in adopting cruelty free testing strategies within the production workflow.

Conflicts of Interest Statement: None

Funding Statement: None

Author contributions

Subhadra Dravida: Conceptualization, reviewing, writing, Vasanthi Dasari: experimental design, methodology and writing, Paparao Bolimera and Swati Shukla: Wet lab experiments and data collection, Rajat Goyal: reviewing, logistics of handling biomolecules, Rahul Ganar and Timothy Elwell: Software and validation.

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