

# Sokoto Journal of Veterinary Sciences



(P-ISSN 1595-093X; E-ISSN 2315-6201)

<http://dx.doi.org/10.4314/sokjvs.v21i1.5>



Yusuf et al./Sokoto Journal of Veterinary Sciences, 21(1): 43 - 46.

## Embryonated eggs as an alternative to animals in the determination of median lethal dose (LD<sub>50</sub>) in *bitis* venom

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**Publication History:**  
Received: 11-06-2022  
Revised: 29-10-2022  
Accepted: 19-11-2022

Determination of median lethal dose (LD<sub>50</sub>) is a vital tool adopted by the World Health Organization for pre-clinical assessment of products for use in the management of snakebite envenoming, a condition which is now included among the list of Neglected Tropical Diseases in 2017. The current trend in the determination of LD<sub>50</sub> involves the use of laboratory animals, tens or even hundreds of animals are sacrificed to achieve this goal. This study aimed to find reliable alternatives to this sacrificing of laboratory animals for research purposes. This study investigated the comparative similarities or differences in results obtained from the use of laboratory animals and embryonated eggs in the determination of LD<sub>50</sub> in snake venom research. The median lethal dose (LD<sub>50</sub>) was determined using female mice using the up and down method and Probit method as well as embryonated eggs. There was no statistical difference in the LD<sub>50</sub> of the venom of *Bitis arietans* obtained by the up and down method and that of the conventional probit analysis ( $p \leq 0.05$ ) (0.325 mg/kg [probit] and 0.351 mg/kg [up and down] respectively). There was also no statistical difference in the LD<sub>50</sub> of the venom of *Bitis arietans* by the up and down method, conventional probit method, and by the use of embryonated eggs ( $p \leq 0.05$ ) (0.325 mg/kg [probit], 0.351 mg/kg [up and down], and 0.392 mg/kg [embryonated eggs]). The three methods used produced values of LD<sub>50</sub> that were within the range reported on the Australian snake and venom database of 2007. The results suggest embryonated eggs can conveniently replace the use of laboratory animals in the determination of LD<sub>50</sub> in snake venom research to ease the ethical challenges posed by excessive use of laboratory animals in snake venom research.

**Keywords:** *Bitis arietans*, Embryonated eggs, LD<sub>50</sub>, Probit analysis, Up and down, Venom

### Introduction

The median lethal dose (LD<sub>50</sub>) test was introduced by Trevan (1927) for the biological standardization of dangerous drugs (Depass, 1989). Recently, however, the LD<sub>50</sub> test has been criticized as an unnecessary

waste of resources with animal rights violations. Therefore, efforts have been made to reduce the number of animals used in such tests and to avoid using this test unless required by regulations or in life-

saving research (Depass, 1989; Feroze *et al.*, 2007). In 1984, a new approach to acute toxicity testing was suggested by the British Toxicology Society based on the administration of a series of fixed-dose levels to a minimal animal population (OECD, 2001). LD<sub>50</sub> has gained wide acceptance as a measure of acute toxicity of all types of substances including snake venom. The lethality of venom and venom fractions is measured by the LD<sub>50</sub> test which also provides a baseline for the determination of ED<sub>50</sub> (Sells, 2003). The assessment of toxic and pharmacological properties of snake venoms and their components is based predominantly on animal experiments (Feroze *et al.*, 2007). Several modifications using fewer animals than the classical LD<sub>50</sub> assay have been published (Depass, 1989; Feroze *et al.*, 2007, OECD, 2001). Most researchers however continue to use the conventional method without considering the fact that several laboratory animals will have to be sacrificed. Several alternatives have been suggested, though with their limitations, these include ELISA, *in vitro* haemolytic test, cell culture, tissue culture, etc (Zurlo *et al.*, 1994; Sells, 2003). The limitations of these tests which include precision, reproducibility and accessibility make it necessary to investigate other methods that will provide a better alternative and yet get similar results without endangering animal lives.

## Materials and Methods

### *Milking of snake venom*

Venom was collected by the milking method described by Markfarlane (1967) from 3 adults *Bitis arietans* snakes of both sexes. Briefly, snakes were restrained on the maxillae, the fangs were introduced to a beaker and the snake voluntarily ejected venom into the beaker. The venoms were pooled together and lyophilized using a desiccator with activated silica gel as the desiccant and stored at 4°C until required.

### *Determination of median lethal dose (LD<sub>50</sub>) of venom of bitis arietans using the conventional method*

The stock solution of each of the crude venom was used (10 mg/ml) as the standard venom concentration. Female albino mice were weighed (20 – 25g), labelled and then divided into 5 groups of 6 mice each. Crude venom was administered at a dose rate of 1 mg/kg, 0.5 mg/kg, 0.25 mg/kg, 0.125 mg/kg and 0.0625 mg/kg intraperitoneally to mice in each group respectively. The mice were observed for mortality for 24 hours as described by Depass (1989). The death/survival rate of each group was recorded. The median lethal dose (LD<sub>50</sub>) was calculated using probit analysis as described by Finney (1952).

### *Determination of median lethal dose (LD<sub>50</sub>) of venom of bitis arietans using the up and down method*

The up-and-down method was introduced by Dixon & Mood (1948) and modified by Bruce (1987). In this method, animals were dosed one at a time starting with an estimated LD<sub>50</sub> dose of 10 mg/kg. If the first animal survived, the next animal received a higher dose by a constant multiplicative factor such as 1.3. If the first animal died, the next animal received a lower dose. Doses are usually adjusted by a constant multiplicative factor such as 0.5. The dose for each successive animal is adjusted up or down depending on the outcome of the previous animal.

### *Determination of median lethal dose (LD<sub>50</sub>) of venom of bitis arietans using embryonated chicken eggs*

Fertile hen eggs incubated for less than 10 days do not have a complete nervous reflex system and therefore cannot experience pain (Rosenbruch, 1989). The eggs have a vascularised yolk sac membrane with normal blood circulation and display a primitive embryonic beating heart, the arrest of which provides a clear indication for lethality testing. The desiccated venom of *Bitis arietans* was weighed and reconstituted with phosphate buffer saline to obtain a 1 mg/ml venom concentration. Thirty-five 9-day-old embryonated eggs were candled to ascertain the viability

The 35 9-day-old embryonated eggs were weighed and wiped with 70 % ethanol (analytical grade). For each venom dose of 5 mg/kg, 2.5 mg/kg, 1.25 mg/kg, 0.625 mg/kg, 0.3125 mg/kg, 5 embryonated eggs were assigned to each venom dose and were administered the venom *in ovo* according to their weights. Five eggs were used as controls and were administered 0.2 ml of phosphate buffer saline each. After 24 hrs the eggs were candled to check for viability.

On day 4 of incubation at 37 °C, eggs were broken out of their shells into containers (artificial shells [Plate I]) and incubated for a further 3 days. On day 6, different doses of venom in physiological saline (2 µl) were applied to a 2 mm diameter filter paper disc which was placed over the vitelline vein on the exposed yolk sac membrane of each egg (5 eggs per group) [Plate II and III]. After 6 hours, the number of embryo deaths was recorded from a range of groups, each group have been injected with a different dose of the venom.

The LD<sub>50</sub> from the three groups were statistically analysed and calculated. Results were compared using the Spearman rank correlation coefficient test using Graphpad Prism (version 6.0) for Windows. Values of p<0.05 were considered significant.

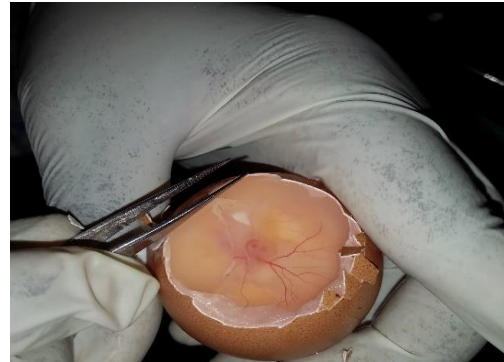
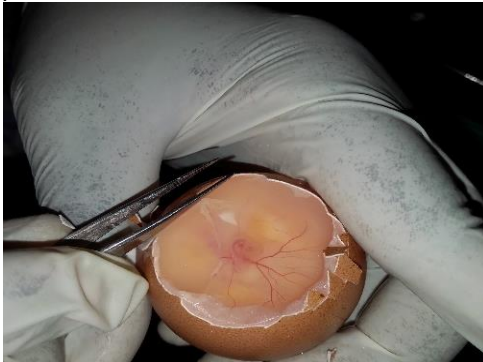
## Results and Discussion

The median lethal dose of *Bitis arietans* venom from this study was 0.325 mg/kg by the conventional probit method, 0.351 mg/kg by the up and down method, and 3.92 mg/kg by the use of embryonated

eggs (Table 1), this was within the range of other previously reported works on the LD<sub>50</sub> of *Bitis arietans* venom of 0.2 – 2.0 mg/kg (Mallow *et al.*, 2003; Anon, 2007) There was no significant difference ( $p \leq 0.05$ ) in the LD<sub>50</sub> of the venom of *Bitis arietans* that was obtained by the up and down method and by the conventional probit analysis ( $p \leq 0.05$ ;  $r = 1.00$ ) (0.325 mg/kg [probit] and 0.351 mg/kg [up and down]



**Plate I:** Preparation of the artificial shell after the first 4 days of incubation  
Prepared container (artificial shell) for incubation of egg yolk



**Plate II:** Egg yolk preparation for further incubation and inoculation  
Incubated hen eggs showing vascularised yolk sac membrane with a normal blood circulation



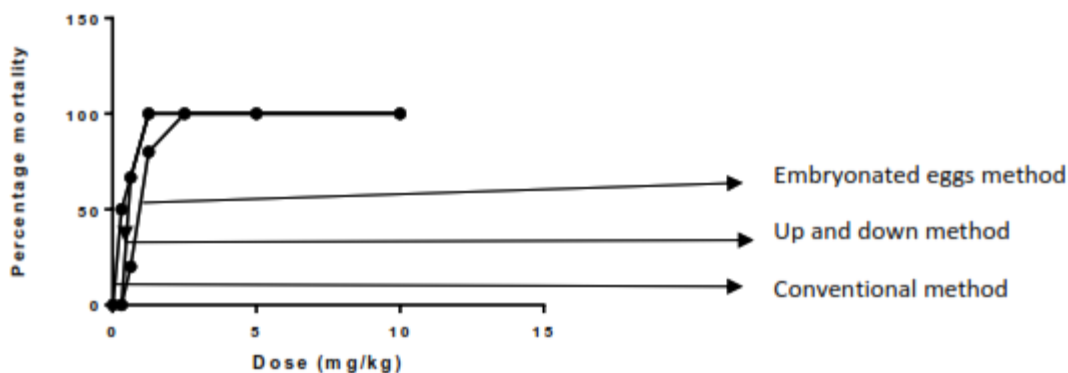
**Plate III:** Egg yolk in artificial shell for further incubation and inoculation  
The exposed vitelline vein in an artificial shell for monitoring embryonic death

respectively). There was also no statistical difference in the LD<sub>50</sub> of *Bitis arietans* venom that was obtained by the up and down method, conventional probit method, and by the use of embryonated eggs ( $p \leq 0.05$ ;  $r = 1.00$ ) (0.325 mg/kg [probit], 0.351 mg/kg [up and down], and 0.392 mg/kg [embryonated eggs]) (Figure 1). Sells *et al.* (1997) and Sells 2003 also reported a strong correlation between results obtained from mouse inoculation and that of embryonated eggs. Depass (1989) reported an excellent agreement in the LD<sub>50</sub> of toxins using the conventional method and the up-and-down method. The three methods used produced values of LD<sub>50</sub> that were within the range reported by the Australian Venom and Toxin database and the Swiss Toxins database (SwissProt/TrEMBL, 2014). Based on the results obtained from this study, we concluded that the determination of the median lethal dose of the venom of *Bitis arietans* can be achieved using the conventional, up and down and embryonated eggs methods. Results obtained from the three methods used had no significant difference ( $p < 0.05$ ). The use of embryonated

**Table 1:** Results for median lethal dose (LD<sub>50</sub>) for the venom of *Bitis arietans* using the conventional probit, up and down, and embryonated eggs methods respectively

Method used	Mean $\pm$ SEM of LD <sub>50</sub> (mg/kg)
Conventional	0.325 $\pm$ 0.03298 <sup>a</sup>
Up and down	0.351 $\pm$ 0.03306 <sup>a</sup>
Embryonated egg	0.392 $\pm$ 0.05458 <sup>a</sup>

Mean  $\pm$  SD for three determinations; values with the same superscripts are not significantly different at  $p < 0.005$



**Figure 1:**Comparative LD<sub>50</sub> curve of the conventional, up and down, and embryonated eggs method

eggs is cheaper and more humane since the pain neurones of eggs at that stage are not fully developed. The embryonated eggs can be used as a reliable and reproducible alternative to animal use in the determination of the median lethal dose for the venom of *Bitis arietans*. It was thus concluded that the LD<sub>50</sub> of the venom of *Bitis arietans* could be determined by the use of embryonated eggs which is more ethical with no statistically different from the up and down and conventional method ( $p \leq 0.05$ ) of determination of LD<sub>50</sub>.

#### Funding

Nil

#### Conflict of Interest

The authors declare that there is no conflict of interest.

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