Immunological properties of the chicken egg yolk (IgY) antibodies against Vietnamese cobra *Naja Naja* venom

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Abstract. Venomous snake bite is a common poisonous accident and important medical problem around the world. The snake anti-venom is only type-specific protection to each venom type that was used to immunize. In this study, the chicken egg yolk (IgY) antibodies against the Vietnamese cobra *Naja Naja* venom was developed and evaluated in *in-vitro* (ELISA and SDS-PAGE) and *in-vivo* (hen and mice immunization). In particularly, the LD₅₀ of the Vietnamese cobra *Naja Naja* venom was determined at 1.46 g kg⁻¹ body weight of the Swiss mice. The ISA Brown layers of 16 to 18-week-old were immunized intramuscularly with the Vietnamese cobra *Naja Naja* venom at 0.5 mg kg⁻¹ body weight of the layers. The specific anti-venom antibodies were presented in both serum and egg yolk at 14 days, reached to highest antibody titer at 49 to 56 days and lasted up to 111 days post-immunization. The IgY antibodies purified from the egg yolk revealed potential neutralization and protection of Vero cells and Swiss mice from toxicity of the Vietnamese cobra *Naja Naja* venom. To the best of our knowledge, this study result is the first cobra snake anti-venom developed in laying hens against the Vietnamese cobra *Naja Naja* venom. Further study is needed to whether this specific IgY anti-venom antibodies can be used as an antibody therapy for treatment of venomous snake bites in the future.

1. Introduction

Venomous snake bite, a common poisonous accident, is mostly found in the tropical and subtropical regions of the world [1]. It is estimated that there are around 5.5 million people are bitten by venomous snakes each year [2,3], in which the cobras and viper cause mortality rate of 9% and 0.2%, respectively [4].

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In Vietnam, it is estimated that there are about 200 snake species, in which there are 53 identified poisonous snake species [5]. Each year, there are about 30 thousand people hospitalized because of poisonous snake bite with the most common poisonous species belonging to the cobra and viper species [5]. The numbers of snack bite patients have increased from April to November that related to the offspring season of the snake species.

The specific anti-venom antibody immune-therapy is the most effective treatment for the snack bite patients. The IgG anti-venom antibodies, obtained from the horse’s serum, were usually used commercially in the market. However, these kinds of anti-venom antibodies are all type specific, meaning that the anti-venom antibody against a type of venom may not protect to neutralize the toxicity from the other venom type. Therefore, WHO recommends building an anti-venom serum system for each country that is equivalence to the poisonous snake species exist.

Traditionally, the anti-venom antibodies are normally produced from serum of the large animal such as horses and sheep. However, the use of such mammalian are more complicate and can causes various side effects such as anaphylactic shock, pyrogen reaction and serum sickness [6]. In recent years, productions of the anti-venom antibodies produce from avian egg yolks are increasingly to replace the serum types for therapeutic and diagnosis uses. Previous studies have shown that the laying hens that were immunized with the different kind of venoms can produce specific anti-venom antibodies for the purpose of enrichment and purification [7,8]. In addition, the amount of anti-venom antibodies produces from the egg yolk were higher than the amount of anti-venom antibodies produces from equine serum [7]. The use of hens for antibody production may significantly increasing the hygienic, convenient, and sources of antibodies, therefore, reducing the costs of commercial anti-venom doses.

The yolk antibodies from chicken eggs have been recognized as a very good source of specific antibodies [6]. The advantages of the production of the yolk antibodies include the more production of the specific antibodies, lower cost and easier to handle compared to the mammal models such as horse and sheep. The production of the specific anti-venom antibodies produced in egg yolks were used to neutralize the toxicity of the various snack venoms such as the *Naja haje arabica* (Arabian cobra) in Saudi Arabia [9], the *Naja naja naja* (Indian cobra) in India [10], the *Coral Micrurus* species in Venezuela [11].

Vietnam is a tropical country that locates on the eastern margin of the Indochinese peninsula [12]. In Northern regions, the cobra *Naja Naja* species are known to be highly toxicity and widely distribution. On the other hands, there are a large of number of snack bites hospitalized each year by these species, and therefore, a specific anti-venom antibody type is needed. In this study, for the first time, we aim to develop the chicken egg yolk (IgY) antibodies against the Vietnamese cobra *Naja Naja* venom. We hope that the study results will support to the optimization of an effective cobra anti-venom production in preventing toxicity of cobra snake bites in future.

2. Materials and Methods

2.1. The Vietnamese cobra *Naja Naja* venom

The lyophilized Vietnamese *Naja Naja* snake venoms were obtained from the Institute of Ecology and Biological Resources, Vietnam National University. The venoms were then reconstituted in 0.9% NaCl solution to obtain final concentration of 10 mg/ml and stored at -20°C for further experiments. All the according to the safety reasons of the Ministry of Health, Vietnam.

2.2. Cells and Animals

Vero cells were obtained from the American type culture collection (ATCC, USA) and used to determine the protection of the egg yolk IgY anti-venom antibodies. Vero cells were grown in Dulbecco’s Modified Eagle Medium (DMEM, Gibco BRL Life Technologies, Grand Island, NY, USA) containing 5% fetal bovine serum (FBS; Gibco BRL Life Technologies) at 37°C in present of 5% CO₂ [13].

Sixteen to eighteen-week-old white leghorn hens, weighing 1500-1800 gram, were used for the production of the egg yolk IgY anti-venom antibodies (AVAC, Vietnam). Swiss mice, weighing 18-20
gram, were used to determine the lethal dose of the *Naja Naja* snake venom, as well as to determine the protection of the egg yolk IgY anti-venom antibodies. The hens and mice were maintained in standard facilities according to the instruction of the animal ethics committee of Vietnamese Government.

### 2.3. Determination of the lethal toxicity of the venoms in mice

The LD50 value of the venoms were carried out as previously described by Reed and Muench [14]. Briefly, three groups of the Swiss mice were injected subcutaneously with 0.5 ml containing the venom at different concentrations of 1µg, 1.25µg, 1.5µg, 1.75µg, and 2.0µg per dose. The un-injected mice were used as controls. The LD50 value was calculated according to the instruction of Reed and Muench method.

### 2.4. Hen immunization

Three groups of the hens were intramuscular immunized with the venoms at a dose of less than 50% of the LD50 value that containing the complete Freund’s adjuvant on day 0, followed with incomplete Freund’s adjuvant on day 14, 28, 42 after the primary immunization [11,15]. The un-immunized hens were used as controls. Blood was collected from the wing vein on 0, 7, 14, 21, 28, 35, and 42 days after the primary boost. Sera were prepared by centrifugation at 1,500 rpm for 10 min, aliquoted, and stored at -80°C until use.

### 2.5. Enzyme-linked immunosorbant assay (ELISA)

The experiments were done in triplicate.

### 2.6. Eggs processing and purification of the IgY antibodies

When the antibody titer in serum was almost stable, eggs were collected and maintained at 4°C until processing. The IgY antibodies were purified as previously described by Akita [16]. Briefly, eggs were broken and the yolk was separated and diluted in ultrapure water at ratio 1:10, adjusted pH to 5.5 and stored at 4°C overnight. The mixture was centrifuged at 5000 rpm in 1h at 4°C, and the supernatant was collected. IgY antibodies were precipitated by ammonium sulfate 29% v/v, gentle mix at 4°C in 4 hrs, centrifuged at 8000 rpm in 60 mins. IgY was suspended in PBS and evaluated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by staining with Coomassie Brilliant Blue R-250 (Bio-Rad, Hercules, CA, USA) [15].

### 2.7. Neutralization efficacy of the IgY antibody

The neutralization activity of the IgY antibodies to the Vero cells were carried out as previously described by Venkatesan et al [11]. Briefly, mixing the prepared IgY antibodies with the LD50 of the venom and incubated at 37°C in 1 hr. The mixture was then added onto the monolayer Vero cells at concentration of 10⁶ cells/well in 96 well plate and incubated at 37°C in present of 5% CO₂ in 24 hrs. The Vero cells incubated with and without the same amount of venom were used as control. The number of cell viability was calculated by neutral red as described previously [17].

The neutralization activity of the IgY antibodies to the mice were carried out as previously described by Lee et al [18]. Briefly, mixing the prepared IgY antibodies with the 2x LD50 of the venom and...
incubated at 37°C in 1 hr. The mixture was then injected subcutaneously to the mice in triplicate. The mice were injected with and without venom were used as controls. The numbers of death mice were calculated after 24 hrs injection.

2.8. Statistical analysis
Statistical analysis was performed using the SPSS Statistical Package program (SPSS Inc, Chicago, IL). The p-values at <0.05 was considered as statistically significant.

3. Results and Discussion

3.1. Lethal toxicity of the Vietnamese cobra Naja Naja venom
The LD_{50} was calculated according to the Reed & Muench method. In this study, the venom was used at five concentrations (1; 1.25; 1.5; 1.75 and 2 µg/g of the body weight). The number of death mice after 24 hrs injection was showed in Table 1. In details, at the venom concentration of 1.25 µg/g the death rate was 12.5% and at the venom concentration of 1.5 µg/g the death rate was 57.1%. Therefore, the LD_{50} of the venom was at 1.46, as calculated below:

$$LD_{50} = \{(57.14-50.0)/(57.14-12.5)\} \times (1.25-1.5) + 1.5 = 1.46$$ (µg/g)

The LD_{50} value of the Vietnamese cobra Naja Naja venom in this study is higher the LD_{50} values of the Africa cobra Naja Naja venom at 0.45 µg/g, 0.35 µg/g, and 0.315 µg/g for the subcutaneous, intravenous, and peritoneal injection, respectively [19]. This result is in agreement with the previous study, in which the toxicity of the cobra Naja Naja venoms depended on the life condition of different geographical areas [20].

| Venom concentration (µg) | Death mice (number of death/injection) | Mice with reaction (number of mice) | Accumulated value (number of mice) | Death rate (%) |
|--------------------------|---------------------------------------|-------------------------------------|-----------------------------------|----------------|
|                          |                                       | Death                        | Live | Death | Live |                       |
| 1                        | 0/5                                   | 0                            | 5    | 0     | 12   | 0                       |
| 1.25                     | 1/5                                   | 1                            | 4    | 1     | 7    | 12.5                    |
| 1.5                      | 3/5                                   | 3                            | 2    | 4     | 3    | 57.1                    |
| 1.75                     | 4/5                                   | 4                            | 1    | 8     | 1    | 88.9                    |
| 2                        | 5/5                                   | 5                            | 0    | 13    | 0    | 100                     |

3.2. Production of hen antibodies against the Vietnamese cobra Naja Naja venom
The group of hens were immunized with the doses of venoms. The level of specific antibodies in the hen sera after immunization were measured by ELISA method (Figure 1). As showed in Figure 1, the specific antibodies of the hens against the venoms started appearing in the serum after seven days of the primary boost and significantly increase at the followed boosts. The highest antibody level was measured at days between 49 and 56 post vaccination (OD value is about 0.9) and the antibodies remained high for over 111 days after vaccination (Figure 1). These results indicated the feasibility of raising antibodies against the Vietnamese cobra Naja Naja venom in hen sera as well as transfer to the egg yolks. The same result of the immunologic response was also found when using the Indian cobra Naja Naja venom immunized hens, in which the highest antibody level was at days between 40 and 60 post vaccination, and the antibodies remained high for over 90 days after vaccination [8].
Figure 1. The production of specific antibodies against the Vietnamese cobra *Naja Naja* venom in immunized hens. The sera were at diluted at 12,800 dilution times and analyzed at 492 nm. The arrows indicated at the start, highest, and reduction points of the antibody titers in the immunization period.

3.3. Purification of the anti-venom specific IgY antibody from the egg yolk

In this study, eggs from the immunized hens were collected after 35 days’ post-venom vaccination. The IgY antibodies were enriched by precipitating with ammonium sulfate as above description. The present of the IgY antibodies were evaluated by SDS-PAGE as showed in Figure 2. The IgY antibodies against venom presented three most visible bands of approximately 18, 45, and 65 kDa, respectively (Figure 2, left panel). This result is in agreement with the previous studies, in which the IgY antibody contains a heavy chain of about 65.105 KDa, a light chain of about 18.66 KDa, and a Fab fragment of about 45.395 KDa [21]. The egg yolk antibody levels were found variation as hens’ immunity respond to the venom after post-vaccination (Figure 2, right panel). In which, the IgY antibodies increase significantly after 35 days’ post vaccination, the highest IgY antibodies were found at day 56, and remained high for over 111 days after vaccination. These results indicated that the specific anti-venom antibodies against the Vietnamese cobra *Naja Naja* venom was successful produced and purified.

![Figure 2](image_url)

Figure 2. The specific IgY anti-venom antibodies from the egg yolk. Production of IgY purification were resolved by 10% SDS-PAGE (left panel). (B) The egg yolk IgY antibody level after post vaccination (right panel). The IgY antibodies were diluted at 1/200.

3.4. Neutralization activities of the IgY antibodies to the Vietnamese cobra *Naja Naja* venom
The anti-venom immune-therapy is the only specific treatment against snake bite by neutralizing the effect of the snake venom invasion. The effects of the specific IgY anti-venom antibodies against snake venoms were well described as its neutralizing activities are higher than the horse serum type from 2 to 6 times [22].

In our study, the effects of neutralization activities of the specific IgY anti-venom antibodies against the Vietnamese cobra Naja Naja venom were examined in the Vero cells and Swiss mice. As expected, the specific IgY anti-venom antibodies showed the inhibitory activity by against the venom at 2× LD$_{50}$ in both Vero cells (Figure 3) and Swiss mice (data not shown). The Vero cells incubated with the mixture between the IgY anti-venom antibodies were growth well (Figure 3d, with 92% cell survival) as showed in the controls of the Vero cells incubated with (Figure 3c, with 87% cell survival) and without (Figure a) IgY anti-venom antibody. In contrast, the Vero cells were dead when incubated with the venom (Figure 3b, with 44% cell survival). The mice were found to be protected from the 2× LD$_{50}$ of the venom by pooling with 1ml of the IgY anti-venom antibodies. These results indicated that the IgY anti-venom antibodies were able to neutralize the Vietnamese cobra Naja Naja venom.

Figure 3. The effect of specific IgY anti-venom antibodies in protection of vero cells from the toxic of Vietnamese cobra Naja Naja venom. (a) Vero cells only. (b) Vero cells were cultured in present of Vietnamese cobra Naja Naja venom. (c) Vero cells were cultured in present of IgY antibodies. (d) Vero cells were cultured in present of Vietnamese cobra Naja Naja venom and IgY antibody mixture.

4. Conclusions
In this study, the specific IgY anti-venom antibodies against the Vietnamese cobra Naja Naja venom was successfully developed and purified. The neutralizing activities of the antibodies were determined by protecting the Vero cells and Swiss mice against toxicity of the 2× LD$_{50}$ of the Vietnamese cobra Naja Naja venom. This result will be helpful for further development of a safety immune-therapy in treatment of the cobra Naja Naja bites in human in future.

Acknowledgment
This research was funded by Nguyen Tat Thanh University, Ho Chi Minh City, Vietnam.

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