Ciência Rural, Santa Maria, v.54:02, e20230042, 2024 http://doi.org/10.1590/0103-8478cr20230042
ISSNe 1678-4596
MICROBIOLOGY

Extraction and quantification of antivenomous antibodies in chicken eggs against scorpion venom

Mehvish Andleeb[1*] **Aleem Ahmed Khan**[1]

[1]Institute of Pure and Applied Biology, Zoology Division, Bahauddin Zakariya University (BZU), 60800, Multan, Punjab, Pakistan. E-mail: mehvish.andleeb01@gmail.com. *Corresponding author.

ABSTRACT: Avian-derived IgY is thought to be the best therapy for scorpion bites concerning low-level side effects. The present study analyzed a hypothesis about the neutralization of scorpion venom *Androcotonus australis* through antibodies produced in the egg yolks of chickens. The venom used for inoculation was obtained from *Androctonus australis* (yellow fat-tailed scorpion) from southern Punjab, Pakistan. The lethal dose of LD_{50} against scorpion venom was calculated in chickens and mice. Safe doses were given to egg-laying chickens to produce IgY antibodies. The antivenom IgY antibodies were extracted from the egg yolks of immunized chicken using the polyethylene glycol (PEG) method. Moreover, IgY was confirmed through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the Ouchterlony double immunodiffusion assay test. The antibody titers were evaluated by the enzyme-linked immunosorbent assay (ELISA). The neutralisation capacity of extracted anti-scorpion antibodies was tested on mice. The calculated LD_{50} of scorpion venom for chicken and mice was 4 mg/kg and 2.5 mg/kg, respectively. SDS-PAGE and Ouchterlony double immunodiffusion confirmed the presence of IgY against scorpion venom. The maximum titer value of specific IgY produced against scorpion venom was 3.5 ug/ml. A concentration of 220 ul/LD_{50} was effective to neutralize 1 mg of scorpion venom. It is suggested that IgY obtained from egg yolks is safe against targeted venom and can be used as an effective alternative to equine IgG antibodies against scorpion envenoming.
Key words: scorpion venom, chicken, Anti-venom, IgY, Neutralization, Mic.

Extração e quantificação de anticorpos entivenenos em ovos de galinha contra veneno de escorpião

RESUMO: Acredita-se que a IgY derivada de aves seja a melhor terapia para picadas de escorpião em relação aos efeitos colaterais. O presente estudo teve como objetivo analisar uma hipótese sobre a neutralização do veneno do escorpião *Androcotonus australis* através de anticorpos produzidos na gema de ovos de galinhas. O veneno usado para inoculação foi obtido de *Androctonus australis* (escorpião amarelo de cauda gorda) do sul de Punjab, Paquistão. A dose letal de LD50 contra veneno de escorpião foi calculada em galinhas e camundongos. Doses seguras foram dadas a galinhas poedeiras para produzir anticorpos IgY. Os anticorpos antiveneno IgY foram extraídos das gemas de ovos de galinhas imunizadas pelo método do polietilenoglicol (PEG). Além disso, a IgY foi confirmada por eletroforese em gel de poliacrilamida e dodecil sulfato de sódio (SDS-PAGE) e pelo teste de imunodifusão dupla de Ouchterlony. Os títulos de anticorpos foram avaliados pelo ensaio imunoenzimático (ELISA). A capacidade de neutralização dos anticorpos anti-escorpião extraídos foi testada em camundongos. A LD50 calculada do veneno de escorpião para galinhas e camundongos foi de 4 mg/kg e 2,5 mg/kg, respectivamente. SDS-PAGE e imunodifusão dupla Ouchterlony confirmaram a presença de IgY contra veneno de escorpião. O valor máximo do título de IgY específico produzido contra veneno de escorpião foi de 3,5 ug/ml. Uma concentração de 220 ul/LD50 foi considerada eficaz para neutralizar 1 mg de veneno de escorpião. Sugere-se que a IgY obtida da gema do ovo seja segura contra o veneno direcionado e possa ser usada como uma alternativa eficaz aos anticorpos IgG equinos contra o envenenamento por escorpiões.
Palavras-chave: veneno de escorpião, frango, anti-veneno, IgY, eutralização, Mic.

INTRODUCTION

Some parts of the world contain highly venomous scorpion species, thus a high rate of mortalities from scorpion stings and because of poor medical assistance in remote areas of the world enhance mortality. Scorpions are the most venomous arachnids (GOMES et al., 2010). There are approximately 2000 described species of scorpions, including 50 species that are dangerous due to their neurotoxic venoms (REIN, 2017). Scorpion venom has anti-inflammatory, anti-microbial effects (HARRISON et al., 2014). Scorpion stings are less life-threatening but more painful. Children and old people are more vulnerable to scorpion stings. However, about 25 species are recognized as a serious threat because of their venom, which can kill humans through stings (POLIS, 1990). All identified species of scorpions have venom, which they use during foraging to paralyse or kill their prey. *Androctonus australis*, commonly known as the yellow fat-tailed scorpion, is found in the deserts of Pakistan. It is one of the world's most perilous species, and its sting is lethal to humans. The venom of this scorpion is so

Received 01.25.23 Approved 04.30.23 Returned by the author 06.14.23
CR-2023-0042.R2
Editor: Rudi Weiblen

powerful that it can kill an adult within two hours. Moreover, its antivenom is not available in Pakistan (SELDON, 2018). It is imported from India and is not too effective against local scorpions in Pakistan.

The first antivenom serum was produced in 1894, and to date, the only medically proven treatment for venom is polyvalent antiserum. The production of antibodies involves the injection of venom into horses, sheep, pigs, rabbits and guinea pigs and the subsequent collection of blood, but demand for antibody production is increasing, as are their uses against venom through new approaches (RUDIGER et al., 2005). Immunoglobulin Y (IgY) is a major antibody in the blood of reptiles, birds and lungfish. This antibody is also found in high quantities in the egg yolks of chicken. IgY is a protein produced by the immune system in response to external particles. Chickens are known to produce antibodies against conserved mammalian proteins and peptides much more efficiently than mammals. This makes the chicken the best choice as a host when working with humans (PEREIRA et al., 2019). Chickens are favoured for antibody production as they lay eggs daily and IgY can easily be extracted from egg yolks, while in the case of mammals such as goats, sheep or horses, extraction of antibodies from blood is a difficult and laborious process. The concentration of IgY is much higher in egg yolks compared to serum. Thus, by immunizing chickens, we have access to a generous source of antibodies solely by collecting eggs (CHUANGJING et al., 2003.

In the last 20 years, chickens have replaced mammals to produce antibodies through eggs as it removes the need to repeatedly take blood samples (POLIS, 1990). Chickens are a cheap and easy source of antibodies (BIZHANOV et al., 2004). Antibody production in chickens has two advantages. First, it reduces the number of animals treated because chickens produce a much higher concentration of antibodies than smaller mammals. Secondly, the purification of antibodies from egg yolks eliminates the need to take blood. However, chickens are less commonly used despite being an excellent producer of antibodies. Limited knowledge is available regarding procedures and applications of IgY in comparison to IgG antibodies (LARSSON et al., 1993).

The current study produced antibodies against scorpion venom in chickens, extracted from eggs. It is a cost-effective method, easy to use and more effective against local species of scorpion in Pakistan. The main objective of the study was the detection and quantification of antivenom antibodies in the yolks of chickens' eggs and the determination of the efficacy and potency of extracted antibodies against scorpion venom in model animals.

MATERIALS AND METHODS

Animals

Domestic chickens (*Gallus domesticus*), weighing 800 grams at 20 weeks old, and mice weighing 20-22 grams were obtained from the market. Fifteen chickens were used for immunization. Ten egg-laying chickens were used as experimental birds and five were taken as control. The experimental birds were divided into two groups, G1 and G2, each containing five birds. Scorpion venom obtained from the locally available scorpion *Androctonus australis* (yellow fat-tailed scorpion), preferably from Cholistan and adjoining areas of Multan (southern Punjab), was preserved in 10% formalin. Adult inbred chickens vaccinated against common poultry diseases were purchased from the government poultry station at the bird market at Ghanta Ghar Chowk, Multan. Swiss albino female mice were maintained at the animal house and used as model animals for testing the antibodies' efficiency. All the animals were maintained at the animal house in the biological garden of Bahauddin Zakariya University (BZU), Multan under standard conditions and were provided with food and water *ad libitum*. All the experimental protocols were approved by the departmental research ethical committee of the Institution of BZU, Multan under university rules (No Biol 4098, dated 02-11-2020).

Venom extraction from scorpion stings

The sting was snipped off from the last segment of each scorpion's tail with a pair of scissors. The venom glands and sting were dried for two days in the sunlight, brought back to the research laboratory of the Institute of Pure and Applied Biology, BZU, Multan, and placed over calcium-chloride in a desiccator. The dried stings and venom glands were ground to a fine powder in the pestle mortar and 0.8 ml saline solution was added in the ratio of one sting to 1 cc of saline solution. Scorpion venom is neurotoxic. The protein concentration of venom was calculated to be 16 ug/ul.

Development of antivenom antibodies in chicken

The first group of chickens, G1, was primed with scorpion venom at a dose rate of 3 mg/kg, while the second group, G2, was injected with scorpion venom at a dose rate of 3.5 mg/kg. The control group was treated with normal saline. No adjuvant was used. Sterile needles and syringes were used to reduce the risk of microbial contamination of injected tissues. Chickens were injected with scorpion venom solution

subcutaneously at a site in the pectoral muscles. A booster dose of venom was given 15 days after the first dose, followed by a second booster dose on the thirtieth day after the first dose.

Isolation and extraction of antivenom antibodies from egg yolks

Isolation and extraction of antibodies from egg yolks were performed by using PEG 6000 (POLSON et al., 1980). The detailed method is attached as a separate sheet. The extracted samples were stored at -20 °C.

Ouchterlony double immunodiffusion

The confirmation of specific antibodies was completed by using the Ouchterlony method (OUCHTERLONY., 1962). First, 8 ug (20 ul) venom and 8 ug (20 ul) antibodies were loaded. Then, the binding of antigens and antibodies was examined.

SDS-PAGE analysis

Using SDS-PAGE, proteins were segregated by their molecular weight larger than 10 KD following the Laemmli method (LAEMMLI, 2011); then, 10 ul molecular markers and 10 ul antibodies were loaded into each well in the gel for SDS-PAGE analysis under reducing conditions.

Indirect ELISA

Antivenom titer was assessed by indirect ELISA. For titer analysis, microtiter plates were first coated with 20-150 ul/well scorpion venom solution with 200 ul/well coating buffer (carbonate and bicarbonate buffer 0.05M, pH 7.4) for one hour at room temperature or overnight at 4 °C (ambient temperature). Wells were washed five times with PBS-T 200 ul/well. After washing, non-binding sites were blocked with 2% nonfat dry milk in PBS-T for two hours at room temperature. After blocking, wells were washed five times and antivenom (IgY) was then added with appropriate dilution and incubated at 37 °C for one hour. After one hour, wells were washed five times with PBS-T, and 200 ul secondary antibodies rabbit anti-chicken IgY conjugated with horse reddish peroxidase (1:1000 dilution) was added to each well and incubated at 37 °C for one hour. After one hour of incubation, wells were washed five times and 150 ul of TMB (MP Biomedicals, Cat. No. 152346) was added to each well. After 30 minutes, with the development of enough colour, the reaction was stopped by adding 3M H_2SO_4 50ul/well. The plate was read by an ELISA reader at a wavelength of 450, and OD_A was obtained.

Protection test to evaluate the efficacy of extracted antibodies (ED 50)

The median lethal dose (LD_{50}) for the venom of the targeted scorpion was evaluated in albino mice weighing 20-22 grams. *In vivo* neutralization of the lethal toxicity of the scorpion venom was assessed by mixing equal volumes of LD_{50} of scorpion venom (2.5 ug/kg) with varying concentrations of neat and diluted IgY and kept for one hour at 37 °C. Four mice were used for each dilution, and a 0.5 ml dose was injected intravenously into each mouse for a fast reaction. Mice were observed for 48 hours and 96 hours (standard time) for survival or death.

RESULTS

Antivenom production by immunising chicken
Venom dose

Both experimental groups were treated with different venom doses, and two booster doses were given to each treated group after 15 days (Table 1).

Collection of eggs

Eggs were collected daily, labeled from day zero to day 45 (up to six weeks) in both groups and stored at 4 °C for future use.

Extraction of IgY by precipitation with polyethylene glycol-6000

IgY antibodies were purified from egg yolks by precipitation with PEG-6000 (Polson method). After purification, IgY was dialysed overnight by

Table 1 - Venom dose (mg) for immunization of chicken G1 represent group 1 of experimental chicken treated with 1st dose and two booster doses of scorpion venom. G2 represent group 2 of experimental chicken treated with three doses. Control group was kept under control conditions. Mg/kg represent the milligram per kilogram.

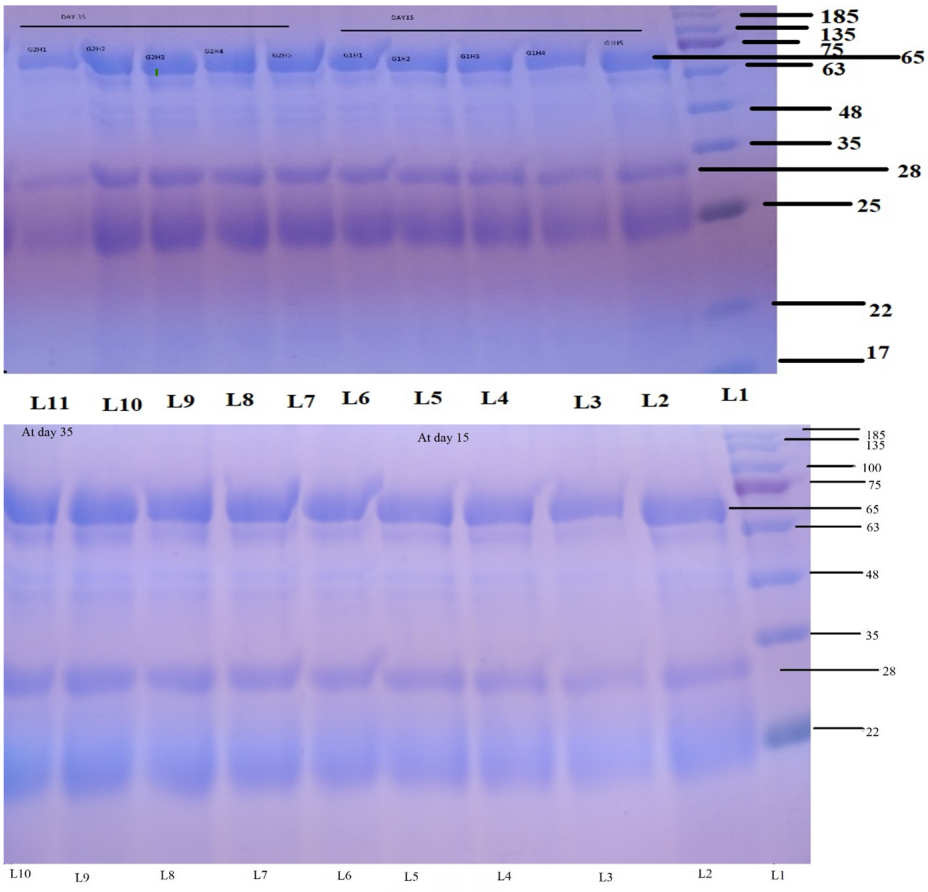
Sr. no.	Experimental groups	1st Immunization dose mg/kg (1st day)	2nd Immunization dose mg/kg (day15)	3rd Immunization dose mg/kg (day 30)
1	G1	3	3.5	4
2	G2	3.5	4	4.5
3	Control	Nil	Nil	Nil

dialysis membrane (MWCO 25 kDa) against 1% saline on a magnetic stirrer with gentle stirring. On the next day, the saline was replaced with 1X PBS of pH 7.4 for one hour. After dialysis, IgY was centrifuged through an ultrafiltration spin column (EZ-10 spin column) to remove contaminants and stored at 4 °C. Purity was checked by protein assay by using a nano-photometer (DESJARDINS et al., 2009).

SDS-PAGE analysis

A reduced SDS analysis was done for confirmation of purified IgY. Treated groups were categorized to run on gel, and purified IgY was taken at days 15, 30 and 45 from both treated groups. Samples were prepared and loaded on gel that was running for three hours. Later, the gel was stained overnight with Coomassie Brilliant Blue R-250 Dye and then destained with a destainer for two to three hours before final observation under an ultraviolet lamp. SDS-PAGE analysis indicated the presence of IgY. Arrows showed two bands, one heavy chain band of 68-65 kDa and one light chain band of 25 kDa. Some additional bands were recognized, which were thought to be the product of the vitellogenin component.

Figure 1- (a, b**)** SDS-PAGE analysis (12% gel under reduced conditions) of IgY antibodies after purification and dialysis: (a) indicates IgY from G1 and (b) indicates the IgY from the G2 group. Lane 1 molecular weight marker, Lanes 2-6 purified IgY on day 15, Lanes 6-11 IgY on day 30. Arrows indicate



(b)

Figure 1 - (a, b). Figure 1.(a,b) SDS polyacrlamide gelelectrophrosis anaylsis (12% gel under reduced conditioin) of IgY antibodies after purification and dialysis. (a) indicate IgY from G1 and (b) indicat the IgY from G2 group. Lane 1 molecular weight marker, lane 2-6 purified IgY at day 15th, lane 6-11 IgY at day 30. Arrows indicates heavy chain (68-65 kda) and light chain (28-25 kda) of IGY antibodies.

heavy chain (68-65 kDa) and light chain (25 kDa) of IgY antibodies.

Double immunodiffusion method

The presence of an opaque precipitant line between antibody and antigen wells was an indication of antigen-antibody interaction. The absence of a precipitant line suggested the absence of any reaction.

Below is the representation of purified antibodies at 35 days for each hen from the G1 and G2 groups, with H representing individual hens. Wells 1-5 contained antivenom lgY of 35 days from five chickens, while the central well contained 8 ug (20 ul) pure scorpion venom (Figure 2).

In figure 3, Well 1 contained antivenom lgY from day 20 from the G1 group, while Well 2 contained antivenom lgY from day 20 from the G2 group. Well 3 contained antivenom lgY from day 30 from the G1 group, while Well 4 contained antivenom lgY from day 30 from the G2 group. Well 5 contained antivenom lgY from day 45 from the G1 group, while Well 6 contained antivenom lgY from day 45 from the G2 group. The central well contained 8 ug (20 ul) of pure scorpion venom. The above picture indicates that no precipitation lines were observed in the plate containing the venom and IgY extracted from the control group chicken, while all experimentally treated plates showed purified antibodies from immunized chickens.

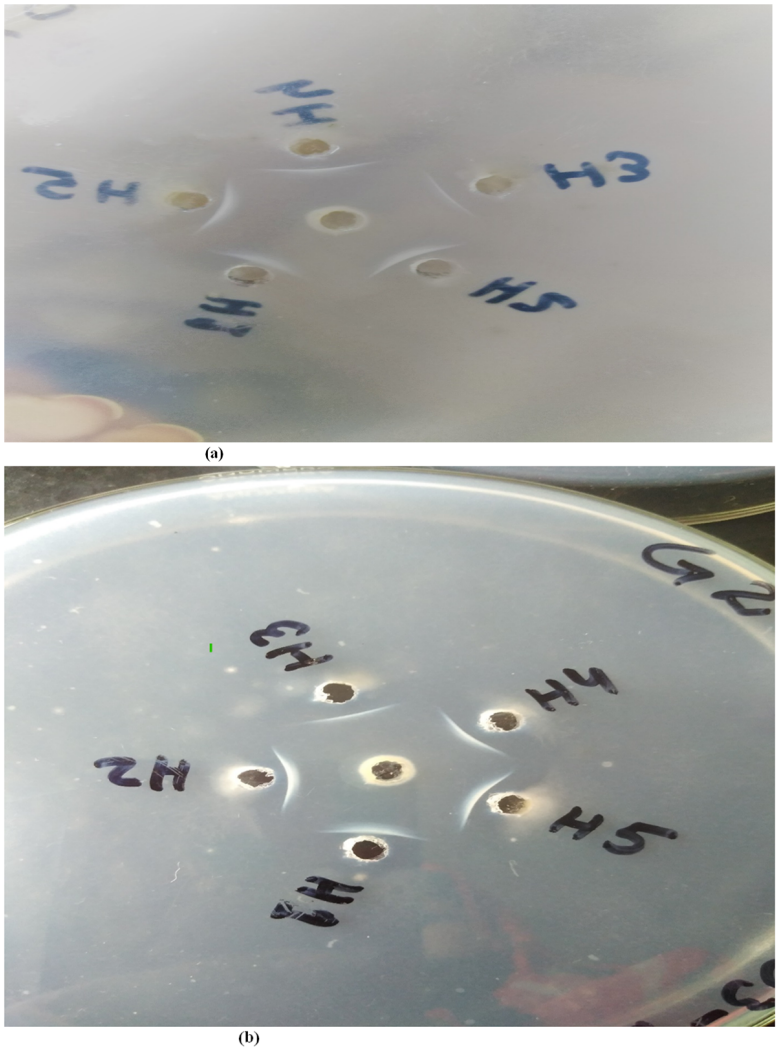


Figure 2 - (a, b) Detection of antivenom lgY against yellow fat tailed scorpion by double immunodiffusion in (a) G1group and (b) G2 group at day 35th.

Analysis of ELISA

Antivenom titer was assessed by indirect ELISA in which titers of antivenom were estimated by two methods. The first was through a serial dilution factor and the second was a weekly evaluation of antivenom titers. In the serial dilution method, a 1/2x factor was used to make dilutions. An amount of 16 ug/ml venom was used to coat the wells of ELISA plates and a 1/2x dilution factor was used to evaluate the IgY titers. The first dilution of 1/2x showed the maximum concentration of antivenom. With the gradual increase in dilution, minimum levels of antivenom were observed in both experimental groups. (Figure 4- a, b).

Weekly titer evaluations of IgY

Another method for estimation of antivenom titers was performed in which the titers of antibodies were compared weekly with the control group (Figure 5).

In the graph, the blue line represents the control group while the orange line represents the samples from the G1 group and the grey line indicates the samples from the G2 group. When compared weekly, control bird samples were found with almost zero concentration of antibodies, in contrast to immunized chickens. The G1 group showed an antivenom titer value of up to 3.1 ug/ul in the sixth week. The G2 group received a high dose of venom and showed an antivenom titer value of up to 3.4 ug/ul in the sixth week. This indicated that high venom dose inoculation triggered the production of more antivenom in the G2 group compared to the G1.

Antivenom effectiveness test: protection test

In vivo neutralization of lethal toxicity of scorpion venom was assessed by mixing equal volumes of LD_{50} of scorpion venom (2.5 ug/kg) with different concentrations of neat and diluted IgY and kept for one hour at 37 °C. Four mice were used for each dilution and 0.5 ml of pure venom was injected intravenously into each mouse. Mice were observed for 48 hours and 96 hours to analyse their survival or death, according to standard procedure.

ED 50 was determined in three groups of mice with four mice in each group: 2.5 ug/kg venom (LD_{50}) was added to different concentrations of antivenom (220, 200, 180 ug/ul) and mixtures were injected into each group of mice. The following results were obtained.

A dose of 220 ul antivenom was enough to completely neutralize the toxicity of the LD_{50} venom dose and all the mice survived. The prepared antivenom was safely able to provide 100% survival against venom in the ED50 assessment assay (Table 2).

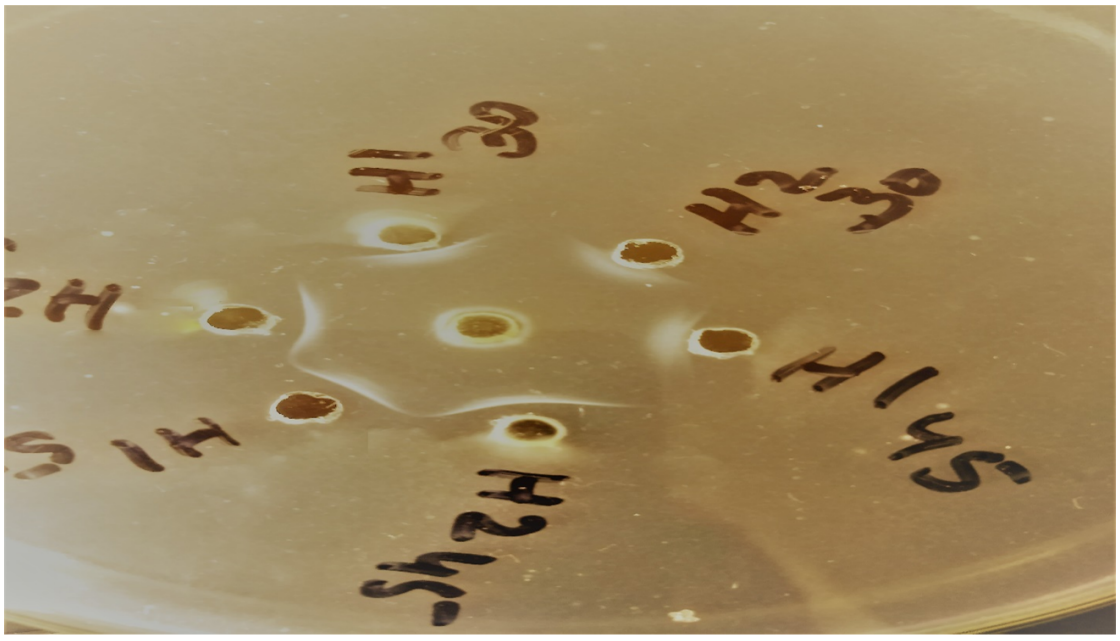


Figure 3 - Detection of anti-scorpion venom lgY against yellow fat tailed scorpion by double immunodiffusion at day 15th, 30th, and 45th days in G2 group.

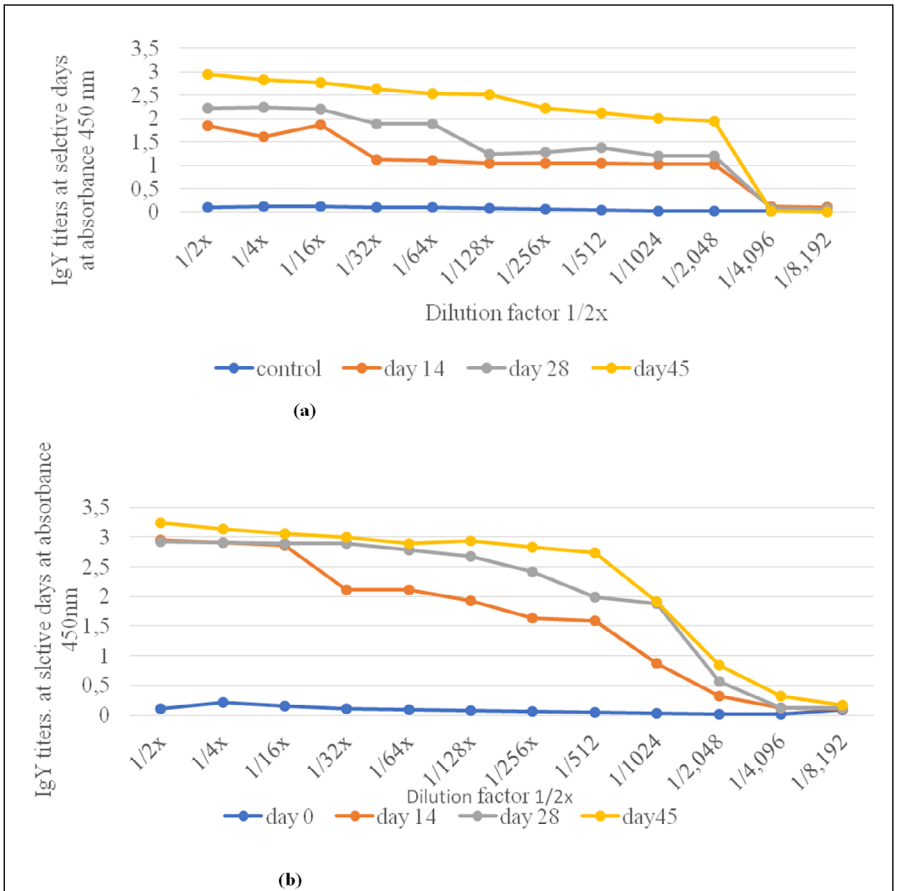


Figure 4 - (a, b) Representation of serial dilution of antivenom titers for the samples of G1 and G2 treated group.

DISCUSSION

The present study pronounced the production of viable and safe antivenom against scorpion venom using the egg yolks of chickens. During the experiment, chickens were immunized with 2-3.5 ug/kg of scorpion venom (antigen) following two booster immunizations (Table 1), and a good immune response was observed as enough IgY was extracted from immunised chicken eggs. The above findings are in line with the studies conducted by AMINA et al. (2018). For IgY antivenom separation, purification and characterisation from egg yolks, PEG was employed as suggested by POLSON et al. (1980).

SDS-PAGE indicated the presence of two bands of IgY in split form; one was 68-65 kDa and the other was 28-25 kDa (Figure 1). Additionally, one extra band of molecular weight 48 kDa was recognised, which may be in result of contamination or impurities resulting from the vitellogenin cleavage product. In the gel, the bands at day 35 and day 45 showed more thickness, which indicated the concentrations of protein antibodies increased to maximum after the second booster dose (Figure 1). A similar trend was obtained when ALVAREZ et al. (2013) employed SDS-PAGE analysis on extracted IgY against *T. Caripitensis* venom, where two bands of 68 kDa and 25 kDa were obtained, with two additional bands of 43 kDa and 35 kDa representing vitellogenin cleavage products. LIU et al. (2017) found two bands, one of 66 kDa and the other of 25 kDa, in IgY samples against snake venom.

The results of the Ouchterlony double immunodiffusion test showed sharp precipitation lines, highlighting the strong binding of antigen with antivenom as described by AGUILAR et al. (2014) and PRABHU et al. (2007). The amount of protein antigen (scorpion venom) used was 1 mg/ml, of which 20 µl was added to the central well. Then, 20 µl antivenom (extracted IgY) was added to each well. The formation of a precipitation line highlighted

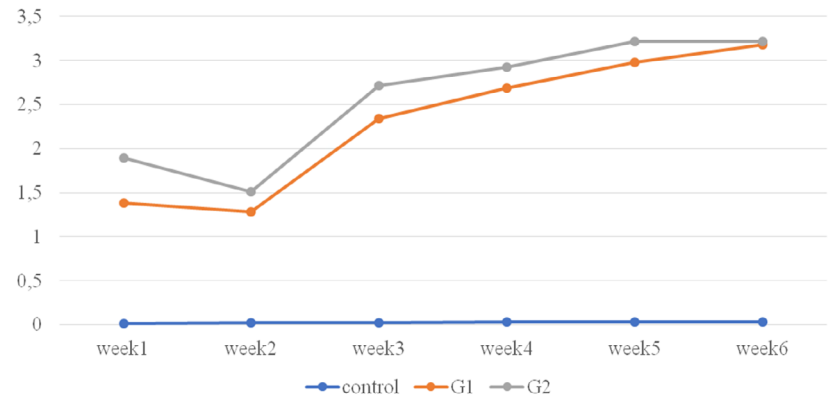


Figure 5 - Titer evaluation of antibodies IgY against scorpion venom from control group and and experimental group (G1 and G2) starting from week 1th to week 6th. Data indicate the continues increase in titre concentarion of IgY of experimental group (orange and gray line) while control group show no change in concentration of IgY (blue line).

the presence of polyvalent IgY immunoglobulins (Figure 2). Figure 2a indicates the samples from the G1 group on day 35, while 2b indicates the samples from the G2 group on day 35. The concentration of antibodies increased after the first and second booster doses, represented by sharp lines (Figure 3). Figure 3 indicates the samples from the G2 group after the first dose (day 15), a first booster dose (day 30) and a second booster dose (day 45). A similar pattern was shown by AGUILAR et al. (2014), where the immunodiffusion test confirmed the presence of special IgY against snake venom. Precipitation lines specified the formation of specific antigen and antibody complexes, where specific IgY can bind with venom present in the central well. Their specific line pattern indicates the interaction pattern between antigens and antibodies. The control group extracted antibodies failed to develop any precipitation line with venom on the test Petri plate.

Indirect ELISA was performed to evaluate the titers of extracted or purified IgY antibodies. Selective samples from days 14, 28 and 45 of both treated groups were evaluated through ELISA assay. An increase in the concentration of IgY antibodies was observed throughout the trial from day 14 to day 45, i.e., the end of the trial, which was assessed by using a protein assay (DESJARDINS et al., 2009).

The maximum concentration was found in the samples on day 45, while the minimum titer was observed on day 14. The samples on day 28 showed a moderate quantity of purified IgY. The titers of antivenom were estimated using two methods: serial dilution factor (Figure 4) and weekly evaluation (Figure 5) of antivenom titers. The results showed that high venom dose inoculation triggered the production of more antivenom in chicken. AMINA et al. (2018) presented similar results while working with the *Androcotonus austalis* scorpion. AGUILAR et al.'s (2014) research exhibited results by working with *Tityus caripitensis* (a venomous scorpion) where the increase in venom dose was directly proportional to antivenom concentration in experimental birds' blood.

Table 2 - Effective Dose 50 values for different venom/antivenom concentrations against scorpion venom.

Antivenom concentration	Venom	---------Number of mice---------			-----Percentage of death-----
mg/ml	Mg/kg	Died	Alived	Total	
220	2.5	0	4	4	0
200	2.5	2	2	4	50
180	2.5	4	0	4	100

A dose of 220 ul antivenom was enough to completely neutralize the toxicity of the LD_{50} venom dose, or 79.1 mg antivenom was capable of neutralizing 1 mg of scorpion venom (Table 2). A similar value was calculated by AMINA et al. (2018), who worked with *Androcotonus austalis* venom with a 221 ul/LD_{50} effective dose (antivenom) against its venom.

According to ALVAREZ et al. (2013), an ED 50 value of about 79.8 mg antivenom against 1 mg of *T. caripitensis* venom in albino mice was recorded, which is low compared with the current study. OKZAN & CARHAN (2009) calculated the neutralization capacity of antivenom against *Androctonus crassicauda* venom in mice and found that one millilitre of *A. crassicauda* antivenom neutralised 940 LD_{50} of *A. crassicauda* venom in mice.

CONCLUSION

We successfully prepared and purified IgY antivenom from immunised chicken eggs and obtained 100% survival in mice without any side effects. Our results indicated that the preparation of antivenom IgY from chickens is important for a high yield of specific antivenoms with minimal side effects. We concluded that IgY may be used as an alternative to antibodies of mammals, especially horses. IgY does not activate the mammalian complement system and does not cause any side effects for humans. Further research is recommended to find applicable uses of IgY in humans so that antivenom could be prepared in large amounts to meet increasing demand to reduce fatalities and side effects. Especially Pakistan is not producing pan-specific antivenom according to demand and has to import supplies from India that are not fully effective and have low efficacy for local scorpion species found in Pakistan. Due to the advantages of IgY technology and its widespread application equally in research and medicine, it is expected to spread on a large scale. IgY is expected to play a cumulative role in research, diagnosis, and immunotherapy in the future.

ACKNOWLEDGMENTS

We would like to thanks to Dr Tahira Ruby Associate professor, Institute of pure and applied Biology Zoology division, Bahauddin Zakariya University Multan for her guidance and support.

DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

BIOETHICS AND BIOSECURITY COMMITTEE APPROVAL

The Animal Ethics Committee of Institution of BZU, Multan, (No, Biol 4098, dated 02-11-2020) approved the present research study protocol.

AUTHORS' CONTRIBUTIONS

Conception and coordination and supervised by DR Aleem Khan. Experimental design, experiment performance, lab analysis and manuscript draft preparation was done by Mehvish Andleeb. All authors critically revised the manuscript and approved of the final version.

REFERENCES

AGUILAR, I. et al. Coral snake antivenom produced in chickens (*Gallus domesticus*). **Inst. Med. Trop.** São Paulo, v.56, n.1, p.61-66, 2014. Available from: <https://doi:/10.1590/S0036-46652014000100009>. Accessed: Sep. 20, 2022. doi: 10.1590/S0036-46652014000100009.

ALVEREZ, A. et al. IGY antibody anti tityus caripitensis venom:purification and neutralization efficacy. **Toxicon**, v.74, p.208-214, 2013 Available from: <https://www.researchgate.net/publication/256331181_IgY_Antibodies_Anti-Tityus_caripitensis_Venom_Purification_and_Neutralization_Efficacy>. Accessed: Aug. 21, 2022. doi: 10.1016/j.toxicon.2013.08.058.

AMINA, S. et al. Development of a new approach of immunotherapy against scorpion envenoming: Avian IgYs an alternative to equine IgGs. **Int Immunopharmaco**, v.1, p.256-26. 2018. Available from: <https://www.researchgate.net/publication/325790269>. Accessed: Aug. 25, 2022. doi: 10.1016/j.intimp.2018.06.013.

BIZHANOV, G. et al. A novel method based on the lithium sulphate of precipitation for purification of chicken eggs yolk IgY, applied to the immunospecific antibodies against Sendi virus. **Scand. J. Lab. Anim. Sci**, v.31, p.121-125, 2004. Available from: <https://doi.org/10.12681/jhvms.15466di>. Accessed: Aug. 26, 2022. doi: 10.12681/jhvms.15466.

CHUANJING, A. et al. Production and characterisation of a novel chicken IgY antibody raised against C-terminal peptide from human thymidine kinase 1. **Journal of Immunological Methods**, n.277, p.157-169, 2003. Available from: <https://pubmed.ncbi.nlm.nih.gov/12799048/>. Accessed: Sep. 02, 2022. doi: 10.1016/s0022-1759(03)00062-0.

CONSROE, P. et al. Comparison of a new ovine antigen binding fragment (Fab) antivenin for United States Crotalidae with the commercial antivenin for protection against venom-induced lethality in mice. **Am J Trop Med Hyg**, n.53, v.10, p.475-493, 1995. Available from: <https://pubmed.ncbi.nlm.nih.gov/7485708>. Accessed: Sep. 12, 2022. doi: 10.4269/ajtmh.1995.53.507.

DESJARDINS, P. et al. Microvolume Protein Concentration Determination using the NanoDrop 2000c Spectrophotometer. **J Vis Exp**, v.33. p.1610, 2009. Available from: <https://pubmed.ncbi.nlm.nih.gov/19890248>. Accessed: Sep. 28, 2022. doi: 10.3791/1610.

GARY, A. P. **The Biology of Scorpions**. Stanford University Press. 1990. 1176-1177p.

GOMES, A., et al. Anticancer potential of animal venoms and toxins. ***Indian*, J. exp. Biol.**, v.48, n.2, p.93-103. 2010. Available from: <https://pubmed.ncbi.nlm.nih.gov/20455317>. Accessed: Sep. 28, 2022. doi: 10.1177/1535370213513.

HARRISON, P. L., et al. Antimicrobial peptides from scorpion venoms. **Toxicon**, v.88, p.115-137. 2014. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7111748>. Accessed: Sep. 28, 2022. doi: 10.1016/j.toxicon.2014.06.006.

HAU, J., HENDRIKSEN, C. Refinement of Polyclonal Antibody Production by Combining Oral Immunization of Chickens with Harvest of Antibodies from the Egg Yolk. **ILAR**, v.46, n.3, p.294-299, 2005. Available from: <https://pubmed.ncbi.nlm.nih.gov/15953836>. Accessed: Sep. 15, 2022. doi: 10.1093/ilar.46.3.294.

LACERDA, A. B. et al. Scorpion envenomation in the state of São Paulo, Brazil: Spatiotemporal analysis of a growing public health concern. **PLOS ONE**, v.17, n.4, e0266138, 2022. Available from: <https://doi.org/10.1371/journal.pone.0266138>. Accessed: Sep. 21, 2022. doi: doi: 10.1371/journal.pone.0266138.

LAEMMLI, H. F. **SDS-.PAGE Protocol. Bio-101**, 2011. Available from: <https://bio-protocol.org/exchange/protocoldetail?id=80&type=1>. Accessed: Sep. 18, 2022.

LARSSON, A. et al. Chicken antibodies: taking advantage of evolution -- a review. **Poultry Sci**, v.72, p.1807-1812, 1993. Available from: <https://pubmed.ncbi.nlm.nih.gov/8415358>. Accessed: Sep. 15, 2022. doi: 10.3382/ps.0721807.

LIU, J. et al. Preparation and neutralization efficacy of IgY antibodies raised against Deinagkistrodon acutus venom. **J. Venom Anim Toxins Incl Trop Dis**, v.2, p.20-22, 2017. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5379703>. Accessed: Sep. 28, 2022. doi: 10.1186/s40409-017-0112-0.

OUCHTERLONY, O. Diffusion-in-gel methods for immunological analysis. II. **Prog Allergy**, v.6, p.30–154, 1962. Available from: <https://pubmed.ncbi.nlm.nih.gov/14482809>. Accessed: Sep. 15, 2022. doi: 10.1159/000313795.

OZKAN, O., CARHAN, A. The neutralizing capacity of Androctonus cras.sicaudaantivenom against Mesobuthus eupeus scorpion venom. **Bangl. J. Vet. Med**, v.7, n.1, p.259-267, 2008. Available from: <https://pubmed.ncbi.nlm.nih.gov/18625264>. Accessed: Sep. 18, 2022. doi: 10.1016/j.toxicon.2008.06.019.

PEREIRA, P. V. et al. Egg yolk antibodies (IgY) and their applications in human and veterinary health: A review, **Int Immunopharmacol**, v.73, p.293–303, 2019. Available from: <https://pubmed.ncbi.nlm.nih.gov/31128529>. Accessed: Sep. 17, 2022. doi: 10.1016/j.intimp.2019.05.015.

POLSON, A. et al. Isolation of Viral IgY Antibodies from Yolks of Immunized Chicken. **Immunological Communications**, v.9, n.5, p.475-493, 1980. Available from: <https://pubmed.ncbi.nlm.nih.gov/7429529>. Accessed: Sep. 19, 2022. doi: 10.3109/08820138009066010.

PRABHU, R. et al. Extraction and characterization of polyclonal egg yolk antibodies (lgY) in chicken against cobra (Naja naja) venom. **Pakistan Journal of Pharmacology**, v.24, n.2, p.29-36, 2007. Available from: <https://www.ijcmas.com/Archives/vol-2-4/R.%20Selvaraj%20et%20al.pdf>. Accessed: Sep. 14, 2022.

REIN, J. O. **The scorpion files**. Norwegian University of Science and Technology, Høgskoleringen, v.1, p.7491, 2017. Trondheim, Norway. Available from: <http://www.ntnu.no/ub/scorpion-files>. Accessed: Sep. 28, 2022.

RUDIGER, S. et al. Chicken egg yolk antibodies (IgY-technology): a review of progress in production and use in research and human and veterinary medicine. **Alternal lab animal**, v.2, p.129-54, 2005. Available from: <https://pubmed.ncbi.nlm.nih.gov/16180988>. Accessed: Sep. 19, 2022. doi: 10.1177/026119290503300208.

SELDEN, P. A. **Arachnids. Reference Module in Life Sciences**. University of Kansas, Lawrence, KS, United States. 2017.