Biotechnology in Animal Husbandry 31 (1), p 109-122 , 2015
Publisher: Institute for Animal Husbandry, Belgrade-Zemun
ISSN 1450-9156
UDC 591.2
DOI: 10.2298/ BAH1501109S

DEVELOPMENT OF IGY ANTIBODIES FOR CONTROL OF TETANUS

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Original scientific paper

Abstract: Tetanus is a cut and often highly fatal infectious disease that affects both human and animals; the disease caused by exotoxin which produced by *C. tetani*. In the current study, we try to get hyperimmune IgY in chicken egg against tetanus toxin and use it as prophylaxis and therapeutic treatment for tetanus. The obtained IgY titer after inoculation of tetanus toxin in chicken eggs was 1320 limit of flocculation (Lf-eq) after 72 hr. IgY in adose of 4500 Lf-eq can be protect donkey after artificial infection by 1 minimum lethal dose (MLD) of *C. tetani*. While a dose of 30000 Lf-eq IgY intramuscularly two times daily for 2 injections, with 9500 Limit of flocculation Lf-eq IgY intrathecally in subarachnoid space was 100% curative for a donkey which was challenged with 1 MLD of *C. tetani*. Furthermore, IgY was evaluated experimentally in comparison with IgG in mice. IgY has equally efficacy to IgG in prophylaxis and treatment of tetanus.

Key words: Tetanus, IgY, IgG, Treatment.

Introduction

Tetanus, commonly called lock jaw, is a wound infection disease that is usually accompanied by fatal toxaemia. The toxaemia causes contraction of voluntary muscle, mainly those of the face, neck, body, legs and tail. Spasms are the results of steady and prolonged contractions of the affected muscles. Tetanus is caused by a rod-shaped germ, *C. tetani*, which produces an extremely potent toxin. The germs form highly resistant, large terminal spores, which give the organism a particular drum stick appearance. The tetanus germs and spores remain localized at the place of the wound where they enter the body. They multiply and produce the powerful toxin (*Johnston, 1994; Kahn, 2010*).

The disease is more prevalent in Lower Egypt than upper one with an infectivity rate of 28 per million in the former and 4.7 per million in the latter.

Infection is more common among equines showing an infectivity rate of 1.2 % in mules, 0.4% in horses and 0.025 % in asses. The infectivity rate in males was always greater than in females. The financial losses were LE 19,057.880 which considered to be far less than the actual losses (calculated for 10 years) (*El-Nahas, 1962).*The recorded cases of equine tetanus in Egypt were 6660 case between 1980 and 1990 (*Ahmed, 1991*).

Chicken eggs consider an ideal alternative antibody source to mammals, as the IgY in the chicken's blood is transported to the egg and accumulates in the egg yolk in large quantities. The existence of an IgG like molecule in avian eggs (IgY), has been well documented in recent studies and extensive research has been carried out on its characterization, production and purification (*Hodek and Stiborová, 2003).*

The chicken egg yolk antibodies (IgY) have been applied successfully for scientific (*Schade et al., 1997*), diagnostic (*Di Lonardo et al., 2001*), prophylactic (*Almeida et al., 1998*) and (*Sarker et al., 2001*) and therapeutic purposes (*Lemamy et al., 1999*), and veterinarian therapy against bacteria such as enteropathogenic E. coli (*Amaral et al. 2002*). There are several distinct advantages for using chickens to produce polyclonal antibodies over than other animals, the chicken IgY have higher titers, animal-friendly, cheaper, nearly unlimited quantities, contain a larger glycosylation index, and stability (*Gassmann et al., 1990*).

This study aimed to prepare antitetanic antibody (IgY) in chicken egg and evaluate it as prophylactic and therapeutic treatment against tetanus in experimental infected animals

Material and methods

Development, purification and titration of anti-tetanic IgY:

a. Purification and concentration of tetanus toxin

Tetanus toxin was purified in VACSERA laboratory (Egypt) using ammonium sulphate, and concentrated to the required concentration. Crude tetanus toxin of 1350 Lf/ml determined by (Ramon, 1922) was used.

b. Immunization of hens

Hens were immunized with tetanus toxin according to the following hyperimmunization schedule (Table 1) after modification the method of *(Rűdiger et al., 1996*).

Table1.Immunization schedule of hens using tetanus toxin

Inoculation Day	Inoculation dose	Adjuvant
0	10 lf tetanus toxin in 0.25 ml normal saline	0.25 ml CFA
7	20 lf tetanus toxin in 0.25 ml normal saline	0.25 ml IFA
14	30 lf tetanus toxin in 0.25 ml normal saline	0.25 ml IFA
21	50 lf tetanus toxin in 0.5 ml normal saline	0.5 ml Aluminum hydroxide (1mM)
28	100 lf tetanus toxin in 0.5 ml normal saline	0.5 ml Aluminum hydroxide (1mM)
35	200 lf tetanus toxin in 0.5 ml normal saline	0.5 ml Aluminum hydroxide (1mM)
42	400 lf tetanus toxin in 0.75 ml normal saline	0.75 ml Aluminum hydroxide (1mM)
49	500 lf tetanus toxin in 0.75 ml normal saline	0.75 ml Aluminum hydroxide (1mM)
56	500 lf tetanus toxin in 0.75 ml normal saline	0.75 ml Aluminum hydroxide (1mM)
63	500 lf tetanus toxin in 0.75 ml normal saline	0.75 ml Aluminum hydroxide (1mM)
	Blood for serum sample and collecting eggs for purification	

Sampling

At 9^{th} day after the last inoculation two samples were taken, 1 ml blood sample per hen from wing vein and laid eggs were collected daily.

Serum collection

Whole blood was collected in a covered test tube. Then allow the blood to clot by leaving it undisturbed at room temperature. This usually takes 15-30 min. The clot was removed through centrifuging at 1.000-2.000xg for 10 min in a refrigerated centrifuge. The resulting supernatant is the serum. The samples should be maintained at 2-8°C while handling. If the serum is not analysed immediately, the serum should be apportioned into 0.5 ml aliquots, stored, and transported at -20°C or lower. It is important to avoid freeze-thaw cycles because this is detrimental to many serum components. Samples which are haemolysed, icteric or lipemic can invalidate certain tests.

c. Purification of egg yolk IgY

Egg yolk free of egg white obtained from pre-warmed refrigerated eggs was rinsed in triple distilled water and rolled in tissue paper for complete removal of the albumen. After several such washes, the yolk membrane was punctured in a pierced funnel shaped filter paper in glass funnel, allowing the yolk to flow into a

graduated measuring cylinder holding the membrane. The yolk was diluted by adding 9 volumes of pre-cooled distilled water and the pH was adjusted to 5-5.2 with 1M HCl and incubated at 4ºC for 6 to 8 hrs. Following incubation the supernatant was harvested and centrifuged at 3000 x g for 25 min in a refrigerated centrifuge. The resulting immunoglobulin (supernatant) containing filtrates (water-soluble fraction) were collected and estimated for protein concentration by the Modified Biuret and Dumas method (*Dumas, 1971*). The IgY containing water-soluble fractions was purified by the salt precipitation method by titrating against 33% ammonium sulphate solution (Algomhoria co., Egypt) as described by *Hansen et al,. (1998)* in three cycles. The precipitate from the last cycle containing IgY was dissolved in normal saline, dialyzed against the same saline until ammonium sulphate was completely removed (*Gazim and Irena, 2003*).

The Protein concentration of the final suspension containing purified immunoglobulin was estimated using the Modified Biuret and Dumas method (*Dumas, 1971*).

d. Titeration of IgY

Using single radial immunodiffusion test according to the method of **(***Ljungqvist and Lyng, 1987*) and Ramon flocculation test according to the method of (*Ramon, 1922*).

Detection of Clostridium tetani minimum lethal dose

20 ml of *C. tetani* (Harvard strain obtained from Abbasia research institute for veterinary vaccine and antisera production, Egypt) bacteria in its media were centrifuged at 3000 rpm for 20 min to remove media and toxins. The precipitated bacteria suspended in 20 ml PBS saline (PH 7.2). These previous steps repeated 2 times. Tenfold series dilutions of the previous suspension in calcium chloride were prepared to reach 2.5% of each tube (1, 1/10, 1/100, 1/1000, $1/10^4$, $1/10^5$, $1/10^6$, $1/10^7$, $1/10^8$, $1/10^9$). In 10 Swiss mice groups each of five inject 0.2 ml per mouse in the thigh muscles one dilution per one mice group. After 4 days explore the highest dilution group where all mice were dead this is minimum lethal dose.

Evaluation of prophylactic and therapeutic capability of anti-tetanic IgY and anti-tetanic IgG in experimental mice

The mice were divided into four groups (15 mice per group) as in table 2.

Table 2. Proposal therapy of mice groups

Days	Group 1 (IgY prophylaxis)	Group 2 (IgY Therapy)	Group 3 (IgG prophylaxis)	Group 4 (IgY Therapy)
0	1000 IU IgY S/C		1000 IU IgG S/C	
1	2MLD of *C. tetani* in 2.5% $CaCl_2$ I.M. in 0.2 ml, 5 mg metronidazole BID rectally for group 1 and 3			
2	5 mg metronidazole BID rectally		5 mg metronidazole BID rectally	
3	5 mg metronidazole BID rectally	1000 IU IgY subcutaneously plus 10 mg metronidazole BID rectally	5 mg metronidazole BID rectally	1000 IU IgG subcutaneously plus 10 mg metronidazole BID rectally
4-11	5 mg metronidazole BID rectally			
12-16		5 mg metronidazole BID rectally		5 mg metronidazole BID rectally

Evaluation of prophylactic capability of anti-tetanic IgY versus metronidazole in experimental infected mice

The mice were divided into three groups each group 10 mice as in table 3.

Table 3. Proposal of IgY and metronidazole prophylaxis of mice groups

Day	Group1 (IgY prophylaxis)	Group 2 (Metronidazole prophylaxis)	Group 3 (Positive control)
0	1000 IU IgY S/C	5 mg metronidazole BID rectally	-
1	2MLD of *C. tetani* in 2.5% $CaCl_2$ I.M. in 0.2 ml 5 mg metronidazole BID rectally for group 2		
2	-	5 mg metronidazole BID rectally	-
3	-	5 mg metronidazole BID rectally	Death of all mice
4-16	-	5 mg metronidazole BID rectally	-

Prophylactic and therapeutic capability of antitetanic IgY in experimental donkeys

Six donkeys divided into 3 groups; 2 donkeys designed for prophylactic group, 2 donkeys designed for therapeutic group and 2 donkey designed for positive control group. The prophylactic group was injected with 3 ml per donkey of IgY (1500 Lf/ml) I/M and metronidazole in a dose of 25 mg /Kg BW three times daily orally for 7 days. After that, all groups injected with 1 ml of crude *C. tetani* in 2.5% calcium chloride.

Symptoms start to appear in therapeutic and positive control groups at 8^{th}

day. These symptoms start as stiffness causing the donkeys to move reluctantly, head and ears are extended, there is evidence of muscular spasms affecting the muscles of mastication and making eating and drinking difficult. It becomes hypersensitive with the external stimuli (sounds, light, touch), and we can note a hyper salivation.

The treatment starts at 8^{th} days with injection of 20 ml of IgY tetanus antitoxin 1500 Lf/ml I/M for two times daily for 2 injections. After hypnotize donkeys with 10% chloral hydrate in a dose of 100 mg/Kg BW I/V, 7 ml IgY tetanus antitoxin 1500 Lf/ml was directly injected into the subarachnoid space through the atlanto-occipital space after removal of 7 ml of CSF.

In addition to, dissolving metronidazole tablets in 20 ml water in a dose of 25 mg/kg BW and administer it per rectum TID for 10 days. In combination with administration of Diazepam 0.1 mg/kg BW to release muscle stiffness, Vitamin C in a dose of 2 gm I/V per day for 10 days.

Results

The results of preparation, purification and titration of anti-tetanic IgY.

The immunization of the hens with tetanus toxins combined with different types of adjuvant and increasing doses according to the schedule, achieve in the development of protective IgY. The purification of IgY through ammonium sulphate precipitation method gives high immunoglobulin yield with low protein impurity where the total protein of egg yolk before purification was 6.5 mg/ml and after purification was 3.9 mg/ml.

a. The result of single radial immunodiffusion test

Known samples with increasing titres were put in the marginal wells while the unknown sample (anti-tetanic IgY) was put in the central well of Petri dish. The dish was incubated 48 hrs. Precipitation rings appear around all wells. The diameters of the marginal precipitation rings were measured (Table 4).

Table 4. Interpretation of petri dish radial precipitation rings

Well Nr.	Lf	Diameter of precipitation ring
1	240	1.3 cm
2	480	1.6 cm
3	720	1.9 cm
4	960	2 cm
5	1200	2.2 cm
U	Unknown IgY sample (anti-tetanic IgY)	2.3 cm

In figure 1, the data obtained from the experiment was plotted where X axis is the diameter of the precipitation ring and Y is the LF equivalent, then by dropping the unknown sample line we can explore that the unknown sample was 1320 Lf-eq.

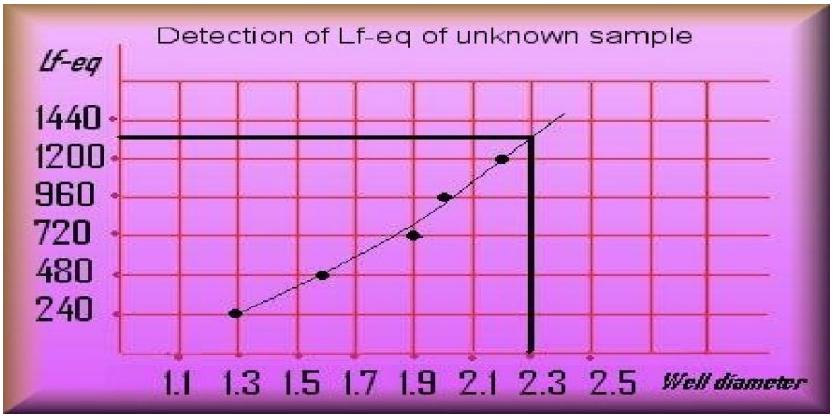

Figure 1. The Ag-Ab precipitation ring diameter was plotted against Known titre to determine unknown sample

b. Results of flocculation test

Results of flocculation test indicated that the first tube that flocculate was test tube No. 6 which contain 1300 Lf is 1 ml purified IgY potency was 1300 Lf-eq.

Results of determination of C. tetani minimum lethal dose

The minimum lethal dose was at the fourth tube with dilution 1/1000 where it's the high dilution that kills all the mice in one group.

Results of evaluation of Prophylactic and therapeutic capability of antitetanic IgY and antitetanic IgG in experimentally infected mice

The mice of therapeutic groups showed signs of at the third day of the experiment which include erected tail, stiffness and rigidity in the injected limb. The therapeutic regime was applied as showed in table 2. The results showed no deaths in the mice of prophylaxis and therapeutic group either with treatment with IgY or IgG. The result indicated that anti-tetanic IgY is as effective as anti-tetanic serum (IgG) originating from equine immunoglobulin.

Results of evaluation of prophylactic capability of antitetanic IgY versus metronidazole in experimental mice

Positive control group results showed that mice were died after 48 hr of experimental infection, while both anti-tetanic IgY prophylaxis mice group and metronidazole prophylaxis mice group still alive till day 10 of the experiment without any tetanus symptoms. The result indicated that anti-tetanic IgY is effective in prophylaxis of experimentally infected mice.

Results of evaluation of prophylactic and therapeutic capability of antitetanic IgY in experimental donkeys

At eighth day of the experiment, symptoms start to appear on both, therapeutic and positive control groups while there was no signs observed on prophylactically IgY treated group.

The prophylactic group showed a dose of 4500 Lf-eq IgY one day before experimental tetanus infection was 100% protective as a prophylactic dose for a donkey of around 100 Kg body weight challenged with 1 MLD of *C. tetani* where no tetanus symptoms appears on this group of animal all over the experiment .

The therapeutic group showed a dose of 30000 Lf-eq IgY I/M BID for one day and 9500 Lf-eq IgY intrathecally was 100% curative for a donkey of around 100 Kg body weight challenged with 1 MLD of *C. tetani* .

The results showed at day 20 of the experiment donkeys of this group starts to open their mouth, and muscle spasms fades but the animals still unable to stand. While at day 27 of the experiment donkeys start to walk and return normal without any spasm but still weak.

The positive control group animal died by respiratory failure due to severe muscle contraction at day 13 of the experiment. The result indicated that anti-tetanic IgY is effective in prophylaxis and treatment of experimentally infected donkeys.

Discussion

Tetanus is a bacterial disease that can affect most animals. Horses are particularly susceptible because of their environment and tendency to incur injuries. Tetanus antitoxin is produced by hyper immunization of donor horses and then harvesting the antibodies. It is used to administer to unvaccinated horses to induce short-lived, immediate, passive protection. The passive immunity usually lasts only 2 to 3 weeks (*Barnett et al., 2001*).

This study was aimed to find a new method of treatment of tetanus using IgY originating from chickens to avoid problems of IgG originating from mammalian origin. Furthermore, the chicken IgY is cheaper, much more in quantity than mammalian antibody and it can be used together with mouse and rabbit antibodies without the danger of cross- reactivity. Secondary antibodies

against chicken IgY's don`t cross react with mammalian IgG's (*Michael et al. 2010*).

There is an increasing interest in the use of chicken egg yolk for polyclonal antibody production for practical and economic reasons (*Bollen et al. 1996; Svendsen et al., 1994; Tini et al., 2002*). The chicken egg yolk antibodies (IgY) have been applied successfully for scientific (*Schade et al., 1997*), diagnostic (*Di Lonardo et al., 2001*), prophylactic (*Sarker et al., 2001; Almeida et al., 1998*) and therapeutic purposes (*Lemamy et al., 1999*).

In this study, hens were immunized with tetanus toxin to hyper-immunization, followed with collection of blood samples and egg at day 9 after last inoculation of tetanus toxin and finally purification of IgY by salt precipitation method using ammonium sulphate solution. Hens could be immunized by tetanus toxin and produce their immunoglobulin in blood which transported to egg yolk in detectable, protective amounts that could be purified and protect other animals by passive immunization (*Marco et al., 2009*).The protein (IgY) concentration in egg yolk after purification with ammonium sulphate determined at week 10 of immunization by spectrophotometer at 280 nm wave length within the absorbance range of 0.2 - 1.5.was 3.9 mg/ml and this agree with the IgY concentration produced with a previous study used ammonium sulphate (*Gazim and Irena, 2003*) where IgY concentration was 3.8 mg/ml. Purification with ammonium sulphate provides high immunoglobulin production, inexpensive and easy to perform.

From a productivity point of view, the yield of IgY obtained by various purification methods is of interest. Literatures reported that the amount of IgY obtained by ammonium sulphate (60% v/v) precipitation was 0.6 mg/ml of egg yolk (Hansen et al., 1998). According to earlier publications (*Hippel and Schleich 1978*) and (*Kabat and Mayer, 1968*) the neutral inorganic salts, such as zinc sulphate and cadmium sulphate induce the precipitation of the proteins.

Although these methods were found effective for IgY purification but had lower IgY production than ammonium sulphate purification.

C. tetani minimum lethal dose in mice was determined for the bacterial culture suspension; the dilution of 1MLD was 1/1000 of the original suspension, this experiment is of great importance to detect the lethality dose of the bacterial strain thus facilitate starting point to the other experiments.

The prophylactic and therapeutic capability of anti-tetanic IgY and anti-tetanic IgG in experimental mice were determined; tetanus symptoms appear on therapeutic groups at the third day of the experiment, the prophylactic and therapeutic effect of both IgG and IgY was identical and effective, this indicate that IgY obtained from tetanus toxin immunized hens has the same effect as IgG that obtained from equine tetanus toxoid hyper immunized serum.

The result of evaluation of Prophylactic and therapeutic capability of anti-tetanic IgY and anti-tetanic IgG in experimental mice are similar to the results

obtained (*Smith and MacIver, 1969*). They found that as the dose of antitoxin was increased, the time at which signs of tetanus first appeared was progressively delayed until, the dose of 500 units. Mice given the largest dose of antitoxin failing to give a high level of protection, (100 units) developed tetanus on average at approximately 9-10 days after injection of the spores. While infected mice with 1 MLD of *C. tetani* in 2.5% calcium chloride and treat them with different units of tetanus antitoxin they found that 1000 units is the best therapeutic dose where all mice in this group were alive.

The prophylactic capability of anti-tetanic IgY versus metronidazole in experimental mice was evaluated as shown in table (3), both anti-tetanic IgY prophylaxis mice group and metronidazole prophylaxis mice group still alive till day 10 of the experiment without any tetanus symptoms, the mode of action of metronidazole is through reduction of their nitro group, however, leads to the production of short-lived cytotoxic intermediates, which finally decompose into nontoxic end products (*Müller, 1983*). That leads to kill *C. tetani* before or shortly after the production of tetanospasmin. While the mode of action of IgY depends on in vivo neutralization of the produced tetanospasmin and modification of effector functions that help the immune system to kill the microorganism.

This experiment agrees with the followings: Metronidazole, a compound widely used in man with minimal side effects, has been shown to be highly active against experimental infections of *C. tetani* and C. welchii in mice (Freeman et al., 1968). Penicillin was used in combination with tetanus antitoxin to reduce the dose of antitoxin required for protection. By preventing multiplication of *C. tetani.*

Other researchers recommend the use of metronidazole (*Cook et al., 2001*) and (*Hsu and Groleau, 2001*). Metronidazole is associated with a better recovery time and a lower mortality rate than penicillin. Penicillin requires adequate blood flow to the site of infection in order to reach effective concentrations. The anaerobic wounds where *C. tetani* thrive often have become devitalized and do not receive enough blood flow. Metronidazole can penetrate devitalized tissue in wounds that penicillin cannot normally reach (*Ahmadsyah and Salim, 1985*).

Prophylactic and therapeutic efficacy of anti-tetanic IgY in experimental infected donkeys was evaluated. At eighth day of the experiment, symptoms start to appear on both, therapeutic and positive control groups, while there was no signs observed on prophylactically IgY treated group.

The results showed that, dose of 4500 Lf-eq of IgY was 100% protective as a prophylactic dose for a donkey of around100 Kg body weight challenged with 1 MLD of *C. tetani* bacteria. While a dose of 30000 Lf-eq IgY intramuscularly BID for 2 shoots, with 9500 Lf-eq IgY intrathecally was 100% curative for a donkey of around100 Kg body weight challenged with 1 MLD of *C. tetani*. Using intrathecal therapy with IgY resulting in the healing of signs, hence this therapy is obviously most beneficial early in the course of illness. In addition, this treatment

was without potential complications as seizures were reported in one of the five horses following intrathecal TAT (*Green et al., 1994*).

Our results indicated that the anti-tetanic IgY had protected both mice and donkeys against experimentally tetanus infection. Therefore, anti-tetanic IgY can be used as an alternative prophylaxis and therapeutic against tetanus infection together with traditional therapy. This study may ensure that the use of anti-tetanic IgY is a new trend in prophylaxis and treatment of animal tetanus in Egypt.

Razvoj IGY antitela za kontrolu tetanusa

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Rezime

Tetanus je česta i veoma fatalna zarazna bolest koja utiče i na ljude i životinje; bolest je uzrokovana egzotoksinom koji proizvodi *C. tetani*. U ovoj studiji, pokušali smo da dobijemo hiperimunih IgY iz kokošijeg jajeta protiv tetanus toksina i koristimo ga kao profilaksu i terapijski tretman za tetanusa. Dobijen IgY titar nakon inokulacije kokošijih jaja tetanusa toksinom bila je 1320 graničnom flokulacije (Lf-eq) posle 72 časa. IgY u dozi od 4500 Lf-eq može zaštititi magarca posle veštačke infekcije sa 1 minimalnom smrtonosnom dozom (MLD) *C. tetani*. Dok doza od 30000 Lf-eq IgY intramuskularno dva puta dnevno za 2 injekcije, sa 9500 graničnom flokulacijom Lf-eq IgY intratekalno u subarahnoidnom prostoru je bila 100% kurativna kod magarca koji je izazvan sa 1 MLD *C. tetani*. Štaviše, IgY je eksperimentalno procenjena u poređenju sa IgG kod miševa. IgY ima jednaku efikasnost/delotvornost kao i IgG, u prevenciji i tretmanu tetanusa.

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Received 22 September 2014; accepted for publication 15 December 2014