

A New Trend in Donkeys Tetanus Treatment Using IgY

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Abstract: Tetanus is a fatal infectious disease that affects both human and animals; it was known that the causative organism is *Clostridium tetani* which is gram positive, anaerobic bacteria. Tetanus is a wound infection disease that is usually accompanied by a fatal toxemia. Tetanus toxin was inoculated into hens to get hyper immune IgY, the titer was 1320 Limit of flocculation (Lf-eq) after 72 days and the titration was approved by Ramon flocculation test and single radial immuno-diffusion test. IgY was evaluated experimentally in donkeys as prophylactic and therapeutic medication. IgY could protect donkeys from 1 minimum lethal dose (MLD) of *Clostridium tetani* bacteria through passive immunization before and after infection. A dose of 4500 Limit of flocculation (Lf-eq) IgY was 100% protective as a prophylactic dose for a donkey of around 100 Kg body weight challenged with 1 minimum lethal dose (MLD) of *Clostridium tetani* bacteria. While a dose of 30000 Limit of flocculation (Lf-eq) IgY intramuscularly two times daily for 2 injections, with 9500 Limit of flocculation (Lf-eq) IgY intrathecally (in subarachnoid space through atlanto-occipital space) was 100% curative for a donkey of around 100 Kg body weight challenged with 1 minimum lethal dose (MLD) of *Clostridium tetani* bacteria. Furthermore, IgY was evaluated experimentally in comparison with IgG in mice. IgY has equally efficacy to IgG in prophylaxis and treatment of tetanus. Thus this trend in tetanus treatment is effective and competitive to IgG treatment.

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1. Introduction

Tetanus is a medical condition characterized by a prolonged contraction of skeletal muscle fibers (or “lockjaw” when the jaw muscles are involved). This occurs despite minimal or no inflammation at the primary site of infection, the primary symptoms are caused by tetanospasmin, a neurotoxin produced by *Clostridium tetani*. There have been 11 strains of *Clostridium tetani* identified. They differ in their ability to produce tetanus toxin, but all strains produce a toxin which is identical in its immunological and pharmacological properties (Johnston 1994; Kahn 2010).

The incidence during the years 1949 to 1958 was studied; deaths among different species are as follows: 50/231 asses, 28/122 mules, 41/173 horses, 4/24 camels, 2/9 cattle, 1/5 buffaloes, 4/11 sheep, 0/3 goats (130/578 total). The disease is more prevalent in the Lower Egypt than upper one with an infectivity rate of 28 per million in the former and 4.7 per million in the latter taking the incidence in 1958 as a base for calculation. Infection is more common among equines showing an infectivity rate of 1.2 % in mules, 0.4 % in horses and 0.025 % in asses (El-Nahas 1962).

The infectivity rate in males is always greater than females (El-Nahas 1962). The financial losses were estimated to be according to recorded cases and deaths: LE 19,057.880 which is considered by

the author to be far less than the actual losses (calculated for 10 years) (El-Nahas 1962).

Chicken eggs present an ideal alternative antibody source to mammals, as IgY in the chicken’s blood is transported to egg and accumulates in 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 (Svendsen et al. 1994; Tini et al. 2002; Schade et al. 1991). The yolk of eggs laid by immunized chicken has been recognized as an excellent source of polyclonal antibodies for over a decade. This simple noninvasive approach presents an appealing alternative to conventional polyclonal antibody production methods. The use of immunoglobulin therapy broadens the arsenal available to combat pathogens in medicine and IgY is a promising candidate (Michael et al. 2010). Furthermore, the chicken IgY have higher titers, double immunostaining is easier to perform, animal-friendly, cheaper, nearly unlimited quantities, contain a larger glycosylation index and stability (Bollen and Hau 1996; Svendsen et al. 1994; Tini et al. 2002). Consequently, this study tries to produce cheaper antibody in chicken as new trend for treatment of tetanus.

2 METHODS

2.1 Production of hen IgY

2.1.1 Experimental birds

10 ISA (Institut de Sélection Animale) brown 5 months old hens, commercially found in Egyptian laying farms, housed as one group in the same place, they are fed on commercially laying chicken feed (18% protein) intended for production from Kahera co. (Egypt).

2.1.2 Toxin

The toxin obtained from VACSERA laboratories (Ministry of Agriculture St., Agha, Egypt). *Clostridium tetani* toxin harvested from Harvard strain which were grown in a modified Mueller and Miller medium, it's measured with Lf (limit of flocculation).

2.1.3 Adjuvant:

1. **Complete Freund's adjuvant (CFA)** from sigma (SIGMA chemical CO. LTD. USA). CFA is a water-in-oil emulsion composed of a light mineral oil, mannide monooleate (a surfactant agent) and heat-killed and dried mycobacterial cells.
2. **Incomplete Freund's adjuvant (IFA)** from sigma (SIGMA chemical CO. LTD. USA). Incomplete Freund's adjuvant (IFA) differs from CFA in that it lacks the killed mycobacterial cells.
3. **Aluminum hydroxide** from El-Nasr co. (El-Nasr pharmaceutical chemical Co.). Aluminum adjuvants are widely used in human vaccines based on their ability to enhance antibody production. However,

the mechanisms underlying these effects remain unknown.

2.1.4 Immunization of hens

Hens were immunized with tetanus toxin according to the following hyper-immunization schedule which describe by John, (1969) and Rüdiger et al, (1996), (Table 1). All these doses were inoculated subcutaneously in the breast area equally divided in the right and left regions

2.1.4.1 Sampling

At 9th day after the last inoculation two samples was 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 analyzed 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 hemolyzed, icteric or lipemic can invalidate certain tests.

Table 1. Immunization schedule of hens using tetanus toxin:

Inoculation day No.	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)
72	<i>Blood for serum sample and collecting eggs for purification</i>	

2.1.5 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 (Algomhoria co., Al America,

Cairo) and incubated at 4°C 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. (Al America, Cairo)

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).

2.1.6 Titration of IgY

The purified IgY was titrated using single radial immuno-diffusion test and Ramon flocculation test.

1. Radial immuno-diffusion

Single radial immuno-diffusion (SRD) method is a method of choice as a simple identity test to confirm presence of tetanus antigen in every final batch of vaccine. The method was published by Melville-Smith et al. (1985) and it is in routine use as an identity test. SRD method can however be used for quantitative measurement of Lf on non-adsorbed purified toxoid and intermediate products. Previous publications suggest that SRD could be used as an alternative to the flocculation test, because the results in the two assay systems do not generally differ by more than 10% (Ljungqvist et al. 1987).

2. Ramon flocculation assay

It's an immunological binding assay in which the flocculation value (Lf) of a toxoid (or toxin) is determined by the number of units of antitoxin which mixed with the sample producing an optimally flocculating mixture. Visible flocculation is formed as a result formation of antigen-antibody complexes. Because calibration of antitoxins in International Units will be assay dependent. Antitoxins used in the Ramon assay must be directly calibrated against the International Reference Reagent (IRR) of tetanus toxoid for flocculation test. The concentration of antitoxin thus determined may be indicated in Limit of flocculation (Lf-eq) uivalents per milliliter (Limit of flocculation (Lf-eq) /ml).

By definitional, 1 Lf is the quantity of toxoid (or toxin) that flocculates in the shortest time with 1 Limit of flocculation (Lf-eq) of specific antitoxin. It is suggested that the Lf-unit of toxoids can be defined not by a relationship to the antitoxin unit but directly by means of a reference toxoid, calibrated in Lf units WHO Expert Committee on Biological Standardization, 1970. The First International Reference Reagent (IRR) of Tetanus Toxoid for Flocculation Test (TEFT) was thus established by the WHO, 1988.

In each of 8 test tubes put 1 ml of purified IgY. Standardized tetanus toxoid add in these test tubes in an ascending manner (300 Lf, 500 Lf, 700 Lf, 900 Lf,

1100 Lf, 1300 Lf, 1500 Lf and 1700 Lf) with PBS as diluents. These test tubes incubated in water bath at 56°C. Then, observe for the first flocculating test tube, this is the Limit of flocculation (Lf-eq).

2.2 Detection of *Clostridiumtetani* minimum lethal dose

20 ml of *Clostridiumtetani* (Harvard strain obtained from Abbasia research institute for veterinary vaccine and antisera production) 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). Repeat these previous steps 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, 1/10⁴, 1/10⁵, 1/10⁶, 1/10⁷, 1/10⁸, 1/10⁹). 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.

2.3 Prophylactic and therapeutic capability of antitetanicIgY in experimental donkeys

5 donkeys with around 100 Kg body wt. divided into 3 groups; 2 donkeys designed for prophylactic group, 2 donkeys designed for therapeutic group and 1 donkey designed for positive control group. The prophylactic group was injected with 3 ml per donkey of IgY (1500 Limit of flocculation (Lf-eq) /ml) intramuscularly and metronidazole in a dose of 25 mg /Kg body weight three times daily orally for 7 days. Following with injection of all groups with 1 ml of crude *Clostridiumtetani* in 2.5% calcium chloride (1 ml equal 1 minimum lethal dose (MLD) for donkey, where 0.2 ml of 1/1000 diluted bacterial suspension is 1 minimum lethal dose (MLD) for 20 gm. mouse.

Minimum lethal dose (MLD) for donkey = $100 \times 0.2 / 0.02 = 1000$ ml of 1/1000 dilution or 1 ml of the crude concentration.

Symptoms start to appear in therapeutic and positive control groups at 8th 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 8th days with; injection of 20 ml of IgY tetanus antitoxin 1500 Limit of flocculation (Lf-eq) /ml intramuscularly for two times daily for 2 injections. After hypnotize donkeys with 10% chloral hydrate in a dose of 100 mg/Kg body weight intravenously, injection of 7 ml IgY tetanus antitoxin 1500 Limit of flocculation (Lf-eq)

/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 tap water in a dose of 25 mg/kg body weight and administer it per rectum TID for 10 days. In combination with administration of Diazepam 0.1 mg/kg body weight to release muscle stiffness, Vitamin C in a dose of 2 gm intravenously per day for 10 days and fluids therapy started when lockjaw was completed and can be stopped when donkeys start to eat.

Positive control donkey was died by severe muscle contraction and respiratory failure at 13th day. Donkeys start to open their mouth, and muscle spasms fades but the animals still unable to stand at 20th day. Donkeys start to walk and return normal without any spasm but still weak 27th day. Prophylactic group were protected and didn't get infection.

3. Result

3.1 Titration of IgY

1. Radial immuno-diffusion test

The IgY was titrated using single radial immuno-diffusion test which showed known titer samples with increasing titers were put in the marginal wells while the unknown titer sample was put in the central well, after 48 hrs. Precipitation rings appear around all wells. The diameters of the marginal precipitation rings were measured (Table 2).

Table 2. Interpretation of IgY titer using single radial immuno-diffusion test

Well No.	Lf	Diameter of precipitation line
1	240	1.3 Cm
2	480	1.6 Cm
3	720	1.9 Cm
4	960	2 Cm
5	1200	2.2 Cm
Unknown IgY sample	?	2.3Cm

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 Limit of flocculation (Lf-eq) (Fig. 1).

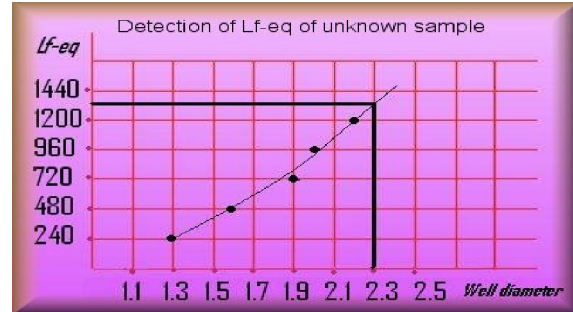


Figure 1: Detection of Limit of flocculation (Lf-eq) of unknown sample

2. Ramon flocculation assay

The first tube that flocculate was test tube No. 6 which contain 1300 Lf, thus 1 ml purified IgY potency was 1300 Limit of flocculation (Lf-eq).

3.2 Result for Detection of Clostridium tetani minimum lethal dose

The minimum lethal dose of *Clostridium tetani* was determined by injection tenfold serial dilution of *Clostridium tetani* suspension in mice. After 4 days explore the highest dilution group where all mice were dead this is minimum lethal dose. The minimum lethal dose was at the fourth tube with dilution 1/1000 (Table 3).

Table 3. Determination of minimum lethal dose (MLD) of *Clostridium tetani*

Dilution of <i>Clostridium tetani</i>	live/ Dead
1	0/5
1/10	0/5
1/100	0/5
1/1000	0/5
1/10 ⁴	1/5
1/10 ⁵	1/5
1/10 ⁶	2/5
1/10 ⁷	3/5
1/10 ⁸	5/5
1/10 ⁹	5/5

3.3 Result of prophylactic and therapeutic capability of anti-tetanic IgY in experimental donkeys

IgY could protect donkeys from 1 minimum lethal dose (MLD) of *Clostridium tetani* through passive immunization before and after infection. A dose of 4500 Limit of flocculation (Lf-eq) IgY was 100% protective as a prophylactic dose for a donkey of around 100 Kg body weight challenged with 1 minimum lethal dose (MLD) of *Clostridium tetani* bacteria. While a dose of 30000 Limit of flocculation (Lf-eq) IgY intramuscularly two times daily for 2 injections, with 9500 Limit of flocculation (Lf-eq) IgY intrathecally (in subarachnoid space through atlanto-occipital space) was 100% curative for a

donkey of around 100 Kg body weight challenged with 1 minimum lethal dose (MLD) of *Clostridium tetani* bacteria.

4. 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 hyperimmunization 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 9th day 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 agrees 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. The IgY was titrated using single radial immunodiffusion test which showed known titer samples after 48 hrs.

The diameters of the marginal precipitation rings were measured where it was 1320 Limit of flocculation (Lf-eq), this titer could protect and treat infected animals. To assure the previous result Ramon's flocculation assay was done with nearly the same result 1300 Limit of flocculation (Lf-eq). The efficiency of obtained IgY was examined as prophylactic and therapeutic treatment in donkeys against tetanus. A dose of 4500 Limit of flocculation (Lf-eq) IgY was 100% protective as a prophylactic dose for a donkey of around 100 Kg body weight challenged with 1 minimum lethal dose (MLD) of *Clostridium tetani* bacteria. While a dose of 30000 Limit of flocculation (Lf-eq) IgY intramuscularly two times daily for 2 injections, with 9500 Limit of flocculation (Lf-eq) IgY intrathecally (in subarachnoid space through atlanto-occipital space) was 100% curative for a donkey of around 100 Kg body weight challenged with 1 minimum lethal dose (MLD) of *Clostridium tetani* bacteria. This proves that hens immunoglobulin are better than mammalian immunoglobulin financially and animal welfare aspects. Using intrathecal therapy with IgY lead to the progression of signs was halted, but not reversed; hence this therapy is obviously most beneficial early in the course of illness. In addition, this treatment is not without potential complications as seizures were reported in one of the five horses following intrathecal tetanus antitoxins (Green et al, 1994).

In the past, penicillin use in combination with tetanus antitoxin to reduce the dose of antitoxin required for protection. By preventing multiplication of *Clostridium tetani*, penicillin should prevent the consequent prolongation of the anaerobic lesions so that antitoxin would be required in the circulation for a shorter time. This possibility was tested by measuring the amount of antitoxin required to protect mice given penicillin 15 hr. after injection of the spores; at this time after infection penicillin alone has no protective effect (Smith 1966).

Other researchers recommend the use of metronidazole (Cook 2001; Hsu 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 *Clostridium 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). Thus, while penicillin and metronidazole may have similar effectiveness in *Clostridium tetani* cultures.

The advantage of IgY production also is the simplicity and ease of purification, and the huge

amount of immunoglobulin production from a few hens.

To confirm the previous result, the experiment was performed in groups of mice with comparing between IgG and IgY. The obtained confirms the result of previous experiment and put IgY equally to IgG in prophylaxis and treatment of tetanus. In a previous study to (Smith and MacIver 1969) where they infected mice with minimum lethal dose (MLD) of *Clostridium tetani* 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 result of the current study gives the same result as (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, with a dose of 500 units, 13 out of 14 infected mice remained well throughout the 28 day observation period. 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.

In conclusion, Chicken eggs present 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. IgY can be used as a preventive and effective treatment for tetanus in donkeys and the donkeys can be tolerating it.

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