Considerations on the stability of IgY antibodies anti-tetanus toxoid

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Abstract
Tetanus is an acute, occasionally a fatal, disease of the central nervous system, caused by the toxin of the tetanus bacterium Clostridium tetani. The current techniques used in the diagnosis and therapeutics of tetanus diseases employ monoclonal antibodies produced in mouse. The use of monoclonal antibodies has several advantages such as homogeneity and specificity. In contrast, a remarkable characteristic of polyclonal antibodies, especially the immunoglobulin class IgY, which is extracted from poultry, is obtained in greater amount than IgG antibodies. Therefore, the alternative production of IgY polyclonal antibodies against tetanus toxoid in chicken can become of great interest for immunology industry in therapeutic and diagnosis. In this work, IgY polyclonal antibodies against tetanus toxoid were produced through immunization of chickens with isolated toxoid and measured by enzyme-linked immunosorbent assay (ELISA). The effect of storage temperature on the stability of chicken IgY was measured through indirect ELISA. Results showed that the IgY antibodies were produced with success and easily isolated. The time and temperature influence was measured through indirect ELISA. Altogether, the results indicate the use IgY polyclonal antibodies purified from chicken as an alternative for the detection of the tetanus toxin with potential applications in diagnosis and treatment of infectious diseases.

Keywords: *IgY antibody – Tetanus toxoid – Antibody stability – ELISA – Clostridium tetani.*

INTRODUCTION

Tetanus is an acute, often a fatal, disease caused by an exotoxin produced by Clostridium tetani[1,2]*. The C.tetani produces two exotoxins, tetanolysin and tetanospasmin. The function of tetanolysin is not known with certainty*[2,3]*. Tetanospasmin is a neurotoxin and causes the clinical manifestations of tetanus*[4]*. Tetanospasmin is one of the most potent toxin known. The estimated minimun human lethal is 2.5 nanograms per kilogram of body weight. C.tetani usually enters*

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the body through a wound[5,6]. Toxins act in several sites within the central nervous system, including peripheral motor and plates, spinal cord, brain and sympathetic nervous system[7]. The typical clinical manifestations of tetanus are caused when tetanus toxin interferes with release neurotransmitters, blocking inhibitor impulses. This leads to unopposed muscle contraction and spasms. Seizures may occur, and the autonomic nervous system may also be affected[4]. For the treatment, serum anti-tetanic is recommended for people with tetanus. The serum can only help removing unbound tetanus toxin. It cannot affect toxin bound to nerve endings[8,9]. A single intramuscular dose of 3.000-5.000 UI is generally recommended for children and adults[10-13].

In generally, this serum is an IgG of equine. The equine antitetanus serum is protein fraction isolated from blood of horses that have been hyperimmunized with tetanus anatoxin or toxin.

Therapeutic proteins in general induce an immune response, especially when administered as multiple doses over prolonged periods. Non-human therapeutic proteins such as equine antitetanus serum induce antibodies by the classical immune reaction and their primary immunogenic factor is the degree of non-self[14]. Therefore, some side effects and reactions are observed. The immediate side effects of serum therapy included fever, chill, and allergic reactions. A delayed toxic reaction of serum therapy was serum sickness, a syndrome characterized by rash, proteinuria, and arthralgia; this occurred in 10% to 50% of patients who received heterologous serum and was probably caused by immune complexes[15].

The antibody industry has many challenges. The production of polyclonal and monoclonal antibodies involve some steps each of which causes distress to the animals involved[16], in addition to other problems such as the aforementioned. In this context the chicken egg yolk antibody, the IgY, emerge as an important alternative. Using chickens as the immunization host brings a number of advantages to antibody production, the most apparent is the (being the) non-invasive collection of antibodies. As described more than 100 years ago, avian maternal antibodies are transferred from serum to egg yolk to confer passive immunity to embryos and neonates[17]. IgY antibody production exploits this by taking the antibodies from the egg yolk, hence the term immunoglobulin of egg yolk (IgY). Another advantage is the enhanced immunogeneity conserved mammalian proteins exhibit in birds due to their phylogenetic distance[18]. The yield of IgY antibodies can be compared to that of IgG antibodies obtained by conventional immunization methods; 200 mg of IgG can be obtained monthly, with approximately 5% constituting the specific antibody. In the case of chicken, approximately 1,500 mg of IgY can be harvested each month, and between 2% and 10% is the specific IgY[19]. Taken together, chicken antibody collection and isolation can be described as noninvasive, rapid and economical[20]. Chickens polyclonal antibodies were produced against a number of antigens and were applied in many different methods for various purposes such as research, diagnostic, therapeutic reagents, besides as a tool for purification or detection of antigens and as a protective agent in passive immunization[21]. The use of IgY in diagnosis is justified by their functional ability in several immune reactions as primary and secondary antibodies. These antibodies have been produced for several applications in diagnosis, such as detection rabies virus in humans, bursal disease, sendai virus, campylobacter fetus diagnosis, and, more recently, improve diagnose HER2 cancer[7,22-24]. The IgY application in therapeutic of some diseases can be used in human and veterinary medicine as shown by Carlander[24]. This includes gastrointestinal pathogens, antibiotic resistant bacteria, virus and others. Some molecular characteristics of the IgY are important for this use, neither activate mammalian complement nor interact with mammalian Fc-receptors that could mediate inflammatory response in the gastrointestinal tract.

In this report, we have developed a method to produce a large amount of chicken egg yolk antibody against tetanus toxin, as well your stability in different conditions, varying time and temperature. Using a simple purification method with carrageenan, the IgY isolated from eggs showed a greater stability in different temperature and time.

MATERIAL AND METHODS

Cell culture and obtainment of Tetanus toxoid

Tetanus toxoid at a protein content of 780 μg/mL were kindly provided by Hertape laboratories, department of microbiology, Juatuba-MG, Brazil. Specify the cell line for the obtainment of the toxoid was maintained in ATCC medium number 1053 (Oxoid CM149).

Tetanus antitoxin

The tetanus antitoxin used in this study was IgY obtained from immunized chickens. The IgY was extracted of serum and egg yolk.

Immunization of chicken and isolation of IgY from egg yolk

Six 12 weeks old with Leghorn hens were immunized with tetanus toxoid. The first immunization was conduced by intramuscular injection with 1 ml (0.5 mg/mL) of antigens (500 μL) in phosphate saline buffer (PBS at pH 7.2) with an equal volume of adjuvant aluminum hydroxide (61,5mg/mL1mM). The second inoculation was conducted at a 1 week interval after the first inoculation. Second inoculations were given through the same route with the 0.5 mg of antigen with two volumes of aluminum hydroxide (500 μL). After the second immunization, a booster inoculation was conducted 3 weeks after the first inoculation without aluminum hydroxide. IgY was extracted from the egg yolk by using 0.1% λ carrageenan solution (Sigma Chemical, Type IV, St Louis, U.S.A). The extract IgY was the filtered through 0.2 μm membrane filter and stored in different time and temperature[18,25].

Storage

After the extraction with carrageenan the antibodies were stored at 41ºC -20ºC and -70ºC for 1, 2, 3 and 6 months before use.

Immunological assays

The titers of IgY were screened by indirect enzyme-linked immunoabsorbent assay (ELISA). The ELISA was adapted of the Farzad, James and McClelland[26], and Gupta and Siber[27]. The plate was cote with 100 μL antigen in the appropriate dilution to each well and then incubated overnight at 4ºC. After the incubation, the plates were washed three times with 150 μL PBST 0.05%. For blocking unspecific binding, the plate was incubated with 0.5% gelatin in PBS, 200 μL/well and then washed as described above. The first antibody (IgY) diluted 1:1000 in PBST was added (50 μL per well), incubated for 1h at 41ºC and washed. The second antibody (anti-chicken enzyme conjugated) diluted 1:3000 in PBST was added (50 μL per well), incubated for 1h at 41ºC, washed three times with PBST and incubated with 50 μL 2,2'-azino-ethyl-benzthizoline-6-sulfonate (ABTS) substrate solution (KPL, Maryland, U.S.A.). After 30 minutes incubation at room temperature, the reaction was stopped by adding 0.1% sodium dodecyl sulphate (SDS) solution and optical density (O.D.) was measured at 490 nm.

Statistical analysis

The antibody titres were compared using Student's test. All values were expressed as mean ± standard deviation and differences with p values <0.05 were considered significant.

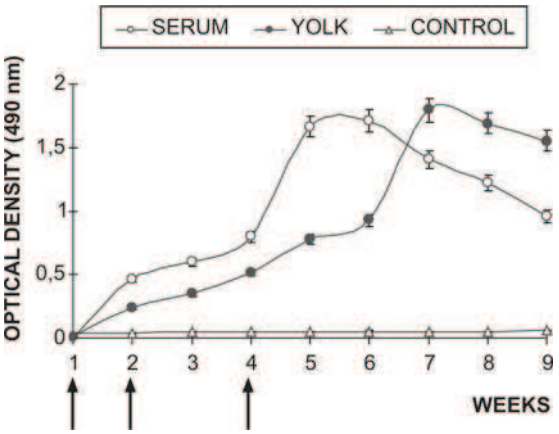
RESULTS

Production of IgY to tetanus toxoid

The development of specifics IgY antibodies against tetanus toxoid has began after the first immunization. The presence of antibodies was detected in the serum and egg yolk by indirect ELISA. The values obtained were compared with the negative control. The evaluation of this assay was made comparing samples tests with negative control. The quality of negative control was determined by coefficient of variation, monitoring the plate variations. All the samples evaluated were positives, therefore, the optical density value were more than of optical density of the negative control.

Eggs were produced up to 30 weeks after the first inoculation of chicken with tetanus toxoid. Although IgY titers were increasing after second

Figure 1. *Kinetics of antibody titres produced by inoculation of tetanus toxoid in laying chickens.*



Notes: the IgY isolated for serum and yolk and tested by indirect ELISA using tetanus toxoid as antigen and expressed as ELISA value (OD at 490 nm); values are the mean of four samples; the vertical bars are due the standard deviation; the black arrow showed the immunizations.

inoculation. The kinetics of IgY production was shown by indirect ELISA. The IgY antibody was extracted of the serum and yolk. The serum and egg yolk were collected after the first inoculation and separated for weeks. Before the extraction, the serum and egg yolk were stored to 41°C, -20°C and -70°C and analyzed by time in these temperatures. In the Figure 1 the immune response of chickens was shown by levels of IgY antibody in the serum and egg yolk in comparison with the control. The poultry efficiency in antibody production can be observed through of the difference of optical density of the samples (negative control, serum and egg yolk). There is a natural trend for the immunoglobulin transfer into eggs in avian. Therefore, the amount of IgY is initially bigger in the serum, and it increases with the booster and time. As expected, the IgY quantify is initially bigger than in the serum. In the sixth week, after the booster immunization, the antibodies concentration reaches its maximum with optical density about 1,71. After that, the natural trend is to occur the transfer of IgY to egg yolk, occurring a quickly inversion. The IgY reaches its maximum on the seventh week with optical density of 1,8, keeping the high concentration for more time and declining more softly.

Effect of storage time and temperature on the stability of chicken IgY

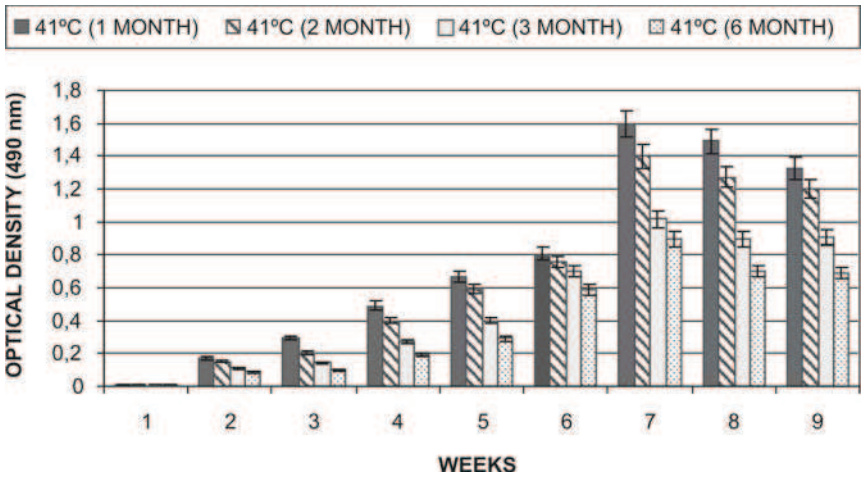
The effect of different storages on the activity of IgY antibody was measured by indirect ELISA. The results have demonstrated that there is a loss of biological activity in relation of time and temperature. Figures 2, 3 and 4 show that the activity of IgY antibody of egg yolk has a decreasing proportional to the time and temperature. The data has been analyzed in accordance with the week of collection, varying the time of storage (1, 2, 3 and 6 months) with fixed temperature (41°C, -20°C and -70°C).

In the Figures 2, 3 and 4 the results demonstrated the same trend, it had a loss of biological activity of antibodies in relation to the time and temperature. When the antibodies were stored at 41°C (Figure 2), occurred a great loss to biological activity. In the -20°C (Figure 3) and -70°C (Figure 4) the loss was more sensible. However, in all the treatments, it did not have significant differences (Table 1). In comparison with the temperatures, was observed that IgY antibodies stored in low temperatures (-20°C and -70°C) conserve its biological activity better than in high temperatures (41°C).

DISCUSSION

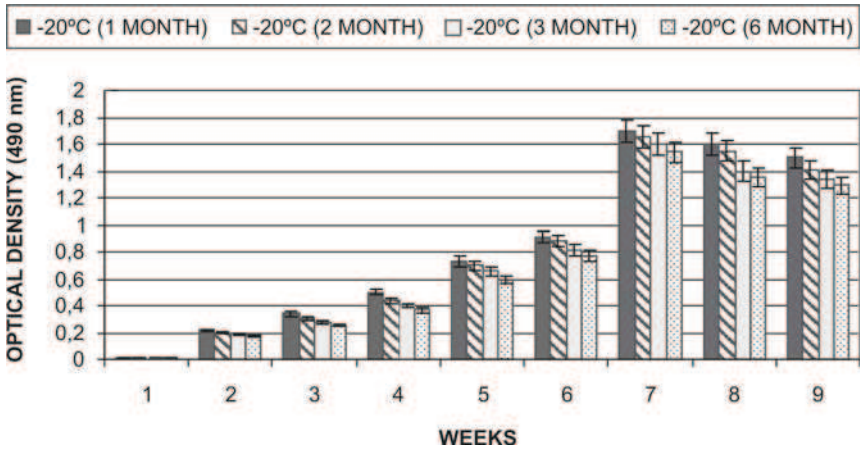
The discovery, in the 19th century, that immune sera were useful in treating infectious diseases[15], Behring and Kitasato established the principle of serum therapy in 1890 against toxin mediated diseases[28,29]. Meanwhile, the serum therapy was abandoned because of its side effects. Over the years, some technologies in antibody production helped in the development and return of serum therapy, principally the hybridoma technology and antibody humanization[30,31]. Nevertheless, more recently, the scientific community has presented some concern about avoiding or minimizing discomfort, distress and pain in the care and use of animals for the production of monoclonal antibodies. Furthermore, the increased prevalence of drug-resistant microorganisms has contributed to industry losses and risk of death in the animals and humans. The development of

Figure 2. *The recoveries of biological activity of IgY against tetanus toxoid when stored at 41°C for 1, 2, 3 and 6 months.*



Notes: the values are means of four samples;the vertical bars indicate the standard deviation.

Figure 3. *The recoveries of biological activity of IgY against tetanus toxoid when stored at 20°C for 1, 2, 3 and 6 months.*



Notes: the values are means of four samples; the vertical bars indicate the standard deviation.

alternatives to use it as therapy and diagnosis of infectious diseases is important. In the last few years, several commercial sources of egg yolk have become available[32].

In this study, we described the development of chicken egg yolk antibody against tetanus toxoid. The antibody levels were measured by indirect ELISA. These antibodies were stored in different conditions varying time and temperature to test the capability of recovery the biological activity. Naturally, the antibodies should be stored for long-term at -20°C or -70°C. In general, the stabilizers use is necessary for better conservation of antibodies. A temperature of 4°C can be recommended for the storage of IgY antibodies over a period of months to a few years, with the addition of 0.02% NaN_3, 0.03% w/v thimerosal or 50µg/ml gentamicin as an inhibitor of bacterial growth[16]. Some works not recommend the freezing at -70°C because it can cause the loss of up to 50% of IgY activity[16].

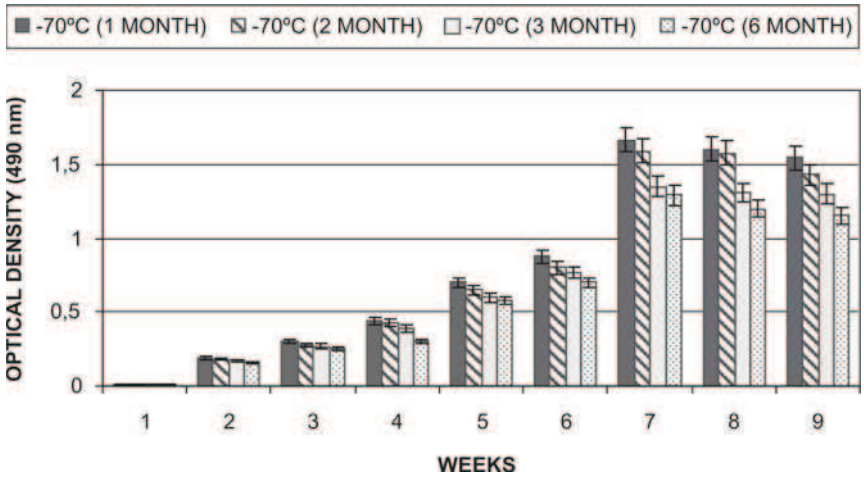
Our group analyzed the biological activity of IgY in three different temperatures of stored (41°C, -20 °C and –70°C) in different times (1, 2, 3 and 6 months) without any type of stabilizers. The results showed that there weren't significant losses. Despite the apparent loss of

Table 1. *Egg yolk antibodies titers of samples stored in different time and temperature.*

TEMPERATURE	1 MONTH	2 MONTHS	3 MONTHS	6 MONTHS
41ºC	1,601[a] ± 0,4	1,400[a] ± 0,5	1,014[a] ± 0,3	0,891[a] ± 0,5
-20ºC	1,701[a] ± 0,5	1,650[a] ± 0,5	1,600[a] ± 0,4	1,540[a] ± 0,5
-70ºC	1,664[a] ± 0,7	1,590[a] ± 0,6	1,350[a] ± 0,5	1,290[a] ± 0,7

Nota: [a] = results with different suffixes differ significantly from results in the same column.

Figure 4. *The recoveries of biological activity of IgY against tetanus toxoid when stored at 70ºC for 1, 2, 3 and 6 months.*



Notes: the values are means of four samples; the vertical bars indicate the standard deviation.

activity of the antibodies stored in 41ºC, the optical density resulting of indirect ELISA was satisfactory.

Since the modern scientific community insists on reducing, refining or even on the replacement of the animal laboratories, there is a need for alternative sources of antibodies that could be produced without distress to the animals. For that and other reasons the present study demonstrated that anti-tetanus toxoid egg yolk antibody can be conserved in different situations with a restoration of biological activity. So, we can observe that there is the possibility to produce the IgY antibody, using your differences above the mammal's antibodies and then storage in low temperatures for many time.

CONCLUSION

A critical examination of the stability properties of the IgY antibodies and other designed antigen-binding proteins reveals that the production and sustained activity of these molecules is more complicated than perhaps is widely appreciated. We aim to use conditions that are common in our laboratory.

In conclusion, the results from the present study indicated that egg yolk antibody are resistant in many different situations, and their stability under these conditions can be due the components of the egg yolk that protected IgY against this effects. In the near future these antibodies will be utilized as therapeutic.

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Considerações sobre a estabilidade de anticorpos IgY anti-tétano toxóide

Resumo

Tétano é uma doença aguda, ocasionalmente fatal, que afeta o sistema nervoso central. Causada pela toxina da bactéria *Clostridium tetani*. As atuais técnicas utilizadas no diagnóstico e terapia da doença empregam anticorpos monoclonais produzidos em camundongos. Os anticorpos monoclonais possuem várias vantagens, como a homogeneidade e a especificidade. Em contrapartida, uma característica notável dos anticorpos policlonais, especialmente a imunoglobulina de classe IgY, que é extraída a partir de aves poedeiras, é a quantidade obtida na extração da gema do ovo. Portanto, a produção de anticorpos policlonais IgY contra o toxóide tetânico em galinhas pode tornar-se de grande interesse para a indústria de imunobiológicos. Neste trabalho, anticorpos policlonais IgY foram produzidos contra o toxóide tetânico através da imunização de galinhas. Esses anticorpos foram isolados e testados pelo método de imunoensaio enzimático indireto (ELISA). Os anticorpos isolados foram armazenados em diferentes temperaturas. O efeito da temperatura de armazenamento sobre a estabilidade da IgY foi medido através de ELISA indireto. Os resultados mostraram que os anticorpos IgY foram produzidas com êxito e facilmente isolados. A influência do tempo e da temperatura, medidos, indicam que os anticorpos isolados são muito resistentes a diversas condições de armazenamento sem a utilização de estabilizadores, demonstrando ser uma importante alternativa para a detecção da toxina do tétano com potenciais aplicações no diagnóstico e tratamento de doenças infecciosas.

Palavras-chave: Anticorpos IgY – Toxina tetânica – Estabilidade – ELISA indireto – *Clostridium tetani*.

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