EVALUATION OF THE FLOCCULATION LIMIT TECNHNIQUE FOR QUALITY CONTROL OF CLOSTRIDIAL VACCINES

[*Avaliação da técnica limite de floculação para o controle de qualidade de vacinas clostridiais*]

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ABSTRACT - This study was carried out to assess and standardize the 'Limit of Flocculation' *in vitro* test to be used in the quality control of vaccine against enterotoxemy. This study used experimental vaccines against enterotoxemy and toxicoid produced from the epsilon toxin of *Clostridium perfringens* type D, previously evaluated by serum neutralization in mice and TCP test. The 'Limit of Flocculation' technique was performed by mixing variable amounts of antitoxin and a fixed value of toxicoid to each tube. It was proposed, in the first tube to flocculate, that its Fl would be the unit of flocculation of the tested sample. The main results indicated that the lower degree of antitoxin detectable both in rabbit and in sheep serum through the 'Limit of Flocculation' technique is equivalent to 10 UI/mL. The TCP and 'Limit of Flocculation' correlation ratio was 99.97%. In conclusion, the Limit of Flocculation is suitable to replace *in vivo* methods for the analysis of epsilon. Nevertheless, the referred technique is less effective to be used for the potency test of vaccines against enterotoxemy in substitution to serum neutralization in mice.

Keywords: Clostridiosis, in vitro methods, potency test, epsilon toxicoid analysis.

RESUMO - Este trabalho foi desenvolvido com o objetivo de avaliar e padronizar o teste *in vitro* Limite de Floculação, para ser utilizado no controle de qualidade de vacinas contra enterotoxemia. Foram utilizadas vacinas experimentais contra enterotoxemia e toxóides produzidos pelo *Clostridium perfringens* tipo D, previamente avaliados por métodos *in vivo*, empregando-se os testes de soroneutralização em camundongo e TCP. A técnica Limite de Floculação consistiu em misturar volumes variados de antitoxina com volumes fixos de toxóide em tubos de ensaio. O primeiro tubo a flocular foi dito como sendo o que continha a quantidade mais próxima de antitoxina equivalente à quantidade de antígeno na amostra e se propôs que o Lf contido no tubo foi unidade de Lf da amostra testada. Os principais resultados obtidos indicaram que o nível mínimo de antitoxina detectável em soros de coelhos e carneiros pela técnica limite de floculação é 10 UI/mL. O coeficiente de correlação, obtido entre os títulos de toxóide pelos métodos TCP e Limite de floculação foi 99,97%. Conclui-se que o Limite de Floculação é satisfatório para substituir os tradicionais métodos *in vivo* utilizados na análise de toxóide epsilon. No entanto, a referida técnica é pouco eficiente para ser utilizada no teste de potência de vacinas contra enterotoxemia, em substituição à soroneutralização em camundongos.

Palavras-Chave: Clostridioses, metodologias *in vitro*, teste de potência, análise de toxóide epsilon.

INTRODUCTION

Enterotoxemy is one of the most important diseases attacking ruminants all over the world. Most animals infected end up dying. A few animals recover from it and remain underdeveloped for the rest of their lives. Therefore, prevention and control measures are necessary to avoid major herd losses. In the profilaxy of enterotoxemy, two measures are stressed, namely, vaccination of all the herd and

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adequate food management. Due to the seriousness of the disease, vaccination is the usual procedure. Since there is a need for vaccines to prevent enterotoxemy, quality control of the products and proper follow-up using scientific parameters must be studied (Titball, 2009). It is clear, therefore, that research should test different methodologies capable of attesting the efficiency of vaccines in a reliable, practical and viable manner.

The limit of flocculation technique is an alternative to substitute for in vivo methods of vaccine quality control, for they are easy to conduct, direct, rapid and economical; it is also convenient to substitute for utilization of animals in analysis of vaccine quality control (Spaun & Lyng, 1970). The technique is based in the antigen-antibody reaction at optimal level and may be utilized to measure antibodies in serum of immunized animals, as well as quantify samples of toxins and/or toxicoids to be used in vaccines.

This study had the purpose of evaluating and standardizing the Limit of Flocculation technique and comparing its sensitivity to serumneutralization in mice, titration of vaccine antibodies from epsilon toxicoid and TCP – Total Combining Power in the evaluation of epsilon toxicoid.

MATERIAL AND METHODS

1. Limit of Flocculation (Lf) for titration of epsilon antitoxin in test serum from rabbits and immunized sheep (Brazilian Pharmacopoeia, 2001).

It was defined as unit of flocculation (Fl) the quantity of toxin that flocculates in the shortest time with a unit of antitoxin in the time it takes for floccules to appear, the constant of flocculation denominated Kf.

The NIBSC epsilon toxicoid was previously titrated with a standard antitoxin containing 100 Lf/mL. In this manner, it was possible to quantify the toxicoid in the Fl unit. The toxicoid was diluted to contain 100 Fl/mL. Next, Ramon's (1922) recommendation for the titration of epsilon antitoxin present in serum of immunized animals was followed. Varied volumes of test serum of immunized sheep were dispensed in a series of seven assay tubes followed by an addition of standard toxicoid containing 100 Fl/mL in each of the tubes. The tubes were immersed in water at 45°C and observed, with the help of magnifying lenses and lighting, in regular intervals up to the appearance of the first floccule. The first, second and third tube containing the mixture that flocculated were recorded, as well as the time necessary for the flocculation to happen in the first tube. The first tube to flocculate was held as having the closest quantity of antitoxin, equivalent to the quantity of antigens in the sample. It was proposed that the Fl in the first tube be the Fl unit of the serum test. The time it took for the first flocculation to occur (Kf) was an indicative of the antigen quality.

To calculate the varied volumes of the sera utilized, the following equation was used:

$$\underset{(mL)}{\text{Serum vol.}} = \frac{\text{Standard Toxicoid Titer(Lf/mL)}}{\text{Stimated Titer(Lf/mL)} \times \text{Serum Dillution}}$$

The proof was read visually and the result consisted of the value estimated for the first tube where the flocculation occurred. In this stage of the study, sera from rabbits and sheep immunized with clostridial vaccines, previously titrated in vivo, were utilized, as shown in Table 1.

Flocculation Limit (Fl) for the evaluation of epsilon toxicoid (Brazilian Pharmacopoeia, 2001).

Firstly, an epsilon antitoxin from rabbit serum was standardized to contain 100 Fl/mL, by utilizing the standard NIBSC epsilon antitoxin containing 100 Fl/mL. The standardization of antitoxin was carried out on the basis of similarity between the units IU and Fl. For this study, the values of Fl adopted were ± 30% of antitoxin titration in IU.

Next, to titrate the text toxicoid, the technique purposed by Ramon (1922) was employed, as described previously, the only difference being the equation for calculation of variable volumes of standard serum utilized, as follows:

$$\underset{(mL)}{\text{Serum vol.}} = \frac{\text{Estimated titer(Lf/mL)} / \text{Toxicoid Dilution}}{\text{Standard serum titer (Lf/mL)}}$$

To evaluate the test's efficiency in the quantification and qualification the epsilon toxicoid, the following samples were utilized: standard NIBSC epsilon toxicoid diluted (D1, D2, D3, D4, D5, D6, D7 e D8) and toxicoids batches T1, T2, T3, T4 e T5, whose titers, obtained through TCP in vivo methodology.

The tests with each toxicoid were carried out 3 times to verify the variability of the test. The reading of the test was conducted visually and the result obtained estimated for the tube where the flocculation firstly occurred.

Table 1 - Epsilon Antitoxin titers, in IU/mL, in serum of immunized rabbit and sheep obtained by serumneutralization in mice.

Rabbit Serum	Titer (UI/mL)		Sheep Serum	Titer (UI/mL)
1	4		1	2
2	1		2	2
3	4		3	5
4	2		4	5
5	3		5	10
6	5		-	-
7	10		-	-
8	10		-	-
9	1		-	-
10	3		-	-

Statistical analysis

The data obtained by the methodologies in vitro and in vivo were analyzed using the F test ($p<0.01$). The correlation between the two methods was assessed by simple linear regression.

RESULTS

1. Verification of the Application of Flocculation Limit by evaluating rabbit and sheep serum immunized with anti-clostridial vaccines.

During this study, it was observed that the minimum antibodies titers present both in the rabbit and sheep serum, detectable through the limit of flocculation, is 10 IU/mL, since only in the serum from sheep obtained in the bleeding of day 5 and serum from rabbit vaccinated with mono-vaccines 7 and 8 it was possible to visualize flocculation, after 2 hours and 40 ± 3 minutes of reaction time.

In the rest of the serum tested, no flocculation was visualized in either of the tubes, thereby suggesting that the limit of flocculation technique is not recommended to measure low antibody concentrations in immunized animals.

2. Verification of application of Flocculation limit in the analysis of epsilon toxicoid

The limit of flocculation limit was first carried out by Ramon (1922), utilizing the standard NIBSC toxicoid in several dilutions, the results being presented in Table 2. The standard serum utilized had epsilon antitoxin titer known as 100 Fl/mL. The sample denominated D1 yielded a result similar to the analysis in vivo (TCP/mL = 640), as may be observed in Table 2. The results obtained in vivo and in vitro are not differentiated by the F test ($p<0.01$).

The time elapsed from the beginning of the reaction until the appearing of the flocculation was not very different among the samples of toxicoids in the varied concentrations. The time it took for it to be visualized varied from 28 to 33 minutes, not following a concentration order. The elevated values of Kf suggest weak power of combination between the antigen and the antibody. Thus, one can relate a slow flocculation with low anti-toxin avidity.

With the results presented in Table 2, it was possible to evaluate the linearity of the method when serialized dilutions of the sample to be tested are employed. Figure 1 illustrates the curve of the linearity obtained from the analysis of limit of flocculation, in different dilutions. From the results presented, it is seen that the limit of flocculation technique is linear in serialized dilutions of the sample, generating a coefficient of correlation 99.96%. The coefficient of correlation found is similar to the one found by Brandi (2007) when evaluating the linearity of the TCP in vivo technique.

When comparing in vivo and in vitro methodologies for the evaluation of epsilon toxicoid, a correlation

Table 2 - NIBSC standard epsilon toxicoid titers, in serialized dilutions, obtained by means of the in vitro Flocculation limit method (Fl/mL).

Standard Toxicoid	Lf / mL	TCP / mL	*In vivo/in vitro*
D1	700	640	1.09
D2	350	320	1.09
D3	175	160	1.09
D4	90	80	1.12
D5	45	40	1.12
D6	20	20	1.00
D7	10	10	1.00
D8	-	< 1	-

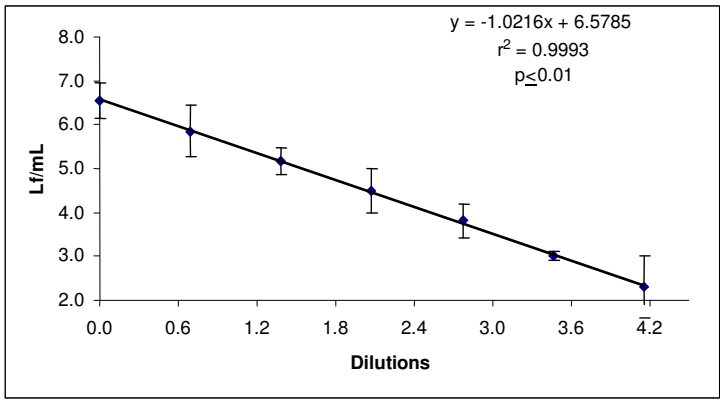

Fig. 1 - Curve of Flocculation limit versus NIBSC toxicoid dilution.

Table 3 - Mean titers of epsilon toxicoid from batch T1, T2, T3, T4 e T5 obtained by means of the Limit of Flocculation method and the relation between the results obtained by means of in vivo and in vitro methods.

Toxicoid	Lf / mL	TCP / mL	*in vivo / in vitro*
NIBSC Standard	-	640	-
T1	2500	2560	1.02
T2	1100	1131	1.03
T3	1300	1500	1.15
T4	500	500	1.00
T5	150	160	1.07

coefficient of 99.97% was found. From the results presented in Table 3, it is seen that the in vivo / in vitro relation reached the maximum of 1.15, thus revealing the proximity of the results obtained by in vivo / in vitro techniques. The flocculation time of the first tube varied in the samples analyzed, obtaining times of 80, 33, 41, 120 and 270 minutes for samples of the batch T1, T3, T2, T4 and T5, respectively.

DISCUSSION

In application of flocculation limit by evaluating rabbit and sheep serum immunized with anti-clostridial vaccines, no flocculation was visualized in most of the tubes. If flocculation occurs in these low concentrations of antibodies, the floccules are not visible to the naked eye or in the time required for such a phenomenon – 12 hours – the time set to carry out the experiment in this study. Next, the tubes were discarded, since it would not be viable to standardize the technique for a long period of time until results were obtained, since the most important is not to perceive flocculation, but the first tube where the formation of floccules occurred. The results show that the limit of flocculation technique is not very sensitive sensitive to measure low numbers of antibodies due to the manner of reading, the direct visualization.

The coefficient of correlation, obtained between the toxicoid ratios through TCP and flocculation limit was of 99.97%, suggesting once more that the in vivo method can be substituted for the in vitro method without loss in result reliability. Corroborating the reports by Spaun and Lying (1970), the relation between the in vivo and in vitro methods was close to 1, suggesting it is a viable alternative for the analysis of epsilon toxicoid. Spaun and Lyng (1970) obtained results equal to 1.40 and 1.36, in average, for the absolute and relative in vivo/in vitro relation, respectively, in tetanic antitoxin titration.

It is seen that the in vivo / in vitro relation reached the maximum of 1.15, thus revealing that in both methods, the antigen / antibody relation should be optimal, so as to culminate when the results appear, be it by observing mice death or formation of flocculates. The degree of antitoxin avidity may influence the in vivo / in vitro relation, since the antitoxin of low avidity flocculates more than neutralizes. Therefore, the higher the in vivo/in vitro relation is to 1, higher its avidity is. It is suggested then that the standard antitoxin utilized in the limit of flocculation technique have high avidity values, so as to reproduce more faithfully what it observed in vivo. The results found in this study were inferior to the ones obtained by Spaun and Lying (1970) and superior to the ones found by Orlans et al. (1960).

The analyses were carried out 3 times and no variation in the results obtained was found. The difference of the results may be attributed to the fact that, in this study, it was employed antitoxins standardized in 100 IU/mL and in fixed reaction temperature of 45 °C. Contrary to what the literature indicates (Weiss & Weiss, 1988), the reagents utilized do not need necessarily to undergo process of purification for flocculation to appear. It was determined that both antitoxin and toxicoid can be incubated in new glass assay tubes without risks. Just to be secure when reading the materials collected, the serum should be filtered in Millipore filter before being used for flocculation, since the blurriness and serum hemolysis can mask the results.

The flocculation time of the first tube varied in the samples analyzed, obtaining times of 80, 33, 41, 120 and 270 minutes for samples of the batch T1, T3, T2, T4 and T5, respectively. Therefore, as in the work conducted by Iwaki et al. (2007), the longest times for flocculation to appear occurred in less concentrated samples. In this manner, T5 sample took 4.5 hours flocculate the first tube, whereas sample T1 flocculate the first tube in 1 hour and 20 minutes. However, the samples of batches T2 and T3 presented flocculation times inferior to the most concentrated sample of batch T1. These results indicate that, besides the low concentration of the toxicoid, the flocculation time reflects its immunogenic quality, implying that the time of flocculation, much more than the concentration, indicates the quality of the antigen (Orlans et al., 1960). Yet, the formulation of vaccines with antigens containing different times of flocculation is necessary to confirm that the immunogenic power of the antigens is directly related to the flocculation time of the toxicoid sample (Bentancor et al., 2009).

This experiment demonstrated that the limit of flocculation technique is a good alternative to substitute for in vivo methods of vaccine quality control, for it is easy, direct, fast and economical. These features make it a very convenient technique to substitute for animal use in the analysis of control quality of vaccines (Spaun & Lyng, 1970), even though Iwaki et al. (2007) reported that analyses involving visual reading are too subjective and present low reproducibility due to great individual variations. This has not been the case in this study, for the analyses were conducted 3 times, by

different technicians, all obtaining the same result. Reading subjectivity can be reduced when duly trained technicians are employed.

Based on the results, it was possible to conclude that the limit of flocculation technique is efficient to evaluate low levels of epsilon antitoxin in sera of rabbits and sheep immunized with clostridial vaccines. This technique is not viable to substitute for serumneutralization in mice when utilized to measure the potency of vaccines against clostridiosis. On the other hand, the TCP in vivo methodology for the control of toxicoid quality aimed at the production of veterinary vaccines can be substituted for the flocculation limit methodology, for the time of flocculation reflects not only the concentration level of the antigen but its quality as well. It is necessary, however, to validate the in vitro, flocculation limit technique presented in this study, for it to substitute for the in vivo methodology of epsilon toxicoid.

The results obtained in this study enable the utilization of the flocculation limit technique, in substitution for the TCP, without compromising the reliability of the results, when it is employed serialized dilutions for the semi-quantification of the toxicoid sample analyzed.

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REFERENCES

Bentancor AB, Halperin P, Flores M, Iribarren F. 2009. Antibody response to the epsilon toxin of *Clostridium perfringens* following vaccination of Lama glama crias. J Infect Dev Ctries. 15;3(8):624-7.

Brandi V.B. 2007. Desenvolvimento e análises de validação de metodologias para o controle de processo de purificação e fabricação de vacinas contra *Clostridium perfringens tipo D*. 2007. 160 f. Tese. (Doutorado em Biotecnologia) - Programa de Pós-Graduação Interunidades em Biotecnologia USP/ Instituto Butantan/IPT, Universidade de São Paulo, São Paulo.

Brazilian Pharmacopoeia, 4 ed. São Paulo: Atheneu. 2001, n. 2.

Iwaki M., Horiuchi Y., Komiya T., Fukuda T., Arakawa Y. & Takahashi M. 2007. Toxoid floculation assay by laser light-scattering. J. Immunol. Methods 10:138-146.

Orlans E.S., Richards C.B. & Jones V.E. 1960. *Clostridium welchii* epsilon-toxin and antitoxin. Immunology 3:28-44.

Ramon, G.A. 1922. Propos du titrage in vitro du sérum antidiphtérique par la flocculation. Compt. Rend. Soc. Biol. 86:813-815.

Spaun J. & Lyng J. 1970. Replacement of the international standard for tetanus antitoxin and the use of standard in the flocculation test. Bull. W.H.O. 42:523-534.

Titball RW. 2009. *Clostridium perfringens* vaccines. Vaccine. 5;27 Suppl 4:D44-7.

Weiss H.E. & Weiss H. 1988. Nachweiss von *Clostridium botulinum*-toxin mittels mikro-wärmekomplementbindungsreaktion. Tierärztl. 43:117-126.