

In vitro potency test for evaluation of *Clostridium perfringens* type D epsilon toxoid

Avaliação *in vitro* da potência de toxoide épsilon de *Clostridium perfringens* tipo D

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ABSTRACT: The control of enterotoxemia caused by the epsilon toxin, produced by *Clostridium perfringens* type D, is based on vaccination with epsilon toxoid. The potency test for this immunogen is conducted using seroneutralization in mice. Here, an *in vitro* test for detection of neutralizing antibodies with Madin-Darby Canine Kidney (MDCK) cells was standardized in order to study alternative methodologies for the potency test. Titers observed in the *in vivo* and *in vitro* seroneutralization tests had a correlation of 99.73%.

KEYWORDS: seroneutralization; mouse; cell culture; bioethics; vaccine.

RESUMO: O controle da enterotoxemia causada pela toxina épsilon produzida por *Clostridium perfringens* tipo D é baseado na vacinação com toxoide. O teste de potência desse imunógeno é realizado utilizando-se a técnica de soroneutralização em camundongos. Objetivando-se estudar metodologias alternativas a essa técnica, padronizou-se um teste *in vitro* para detecção de anticorpos neutralizantes utilizando-se a linhagem celular Madin-Darby Canine Kidney (MDCK). Os títulos observados nas soroneutralizações *in vivo* e *in vitro* apresentaram correlação de 99,73%.

PALAVRAS-CHAVE: soroneutralização; camundongo; cultura de células; bioética; vacina.

This study was conducted according to the ethical principles of animal experimentation established by the Ethics Committee for Animal Experimentation (CETEA/UFMG), protocol n. 40/2008, as determined by the declaration emitted by the Coordinator of the Committee on June 3, 2008.

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Received on: 12/02/2012. Accepted on: 10/22/2013

Enterotoxemia is a widespread disease of domestic ruminants caused by epsilon toxin of *Clostridium perfringens* type D that results in significant economical losses for the meat and dairy industry (LOBATO *et al.*, 2010). The control and prophylaxis are based on good management practices and systematic vaccination. The standard technique proposed by Brazilian legislation for potency tests of clostridial vaccines is seroneutralization (SN) in mice (MAPA, 1997). Despite the known sensitivity and reliability of this potency test in mice, seroneutralization is time-consuming, relatively expensive, and considered ethically debatable by humanitarian groups and researchers aiming to protect animal welfare (METZ *et al.*, 2002).

These bioethical issues have inspired further studies regarding the development of *in vitro* assays that yield quick, reliable results with good sensitivity and specificity at a decreased cost. Therefore, cell culture methods have been regarded as possible alternatives to SN in mice (BORRMANN *et al.*, 2006). This work aimed to standardize an *in vitro* SN test to evaluate the potency of epsilon toxoid and to compare this cell culture-based method to standard animal bioassays.

The production of epsilon toxin was performed as previously described by AZEVEDO *et al.* (1998). A standard serum (National Institute for Biological Standards and Control – NIBSC, UK) with 10 IU/mL of anti-epsilon toxin antibodies was used for epsilon-toxin standardization. Test sera used in both the *in vitro* and *in vivo* SN were obtained from pools of rabbits immunized with clostridial vaccines containing epsilon toxoid and standard serum with different concentrations. Negative control rabbits were inoculated with 0.85% saline solution under the same conditions. These test sera were titrated in mice according to LOBATO *et al.* (2000) at the L+/10 level (BRITISH PHARMACOPEIA, 1998). The use of animals in this study was approved by the Ethics Committee for Animal Experimentation (CETEA), Federal University of Minas Gerais, nº 40/2008. The cell line used was Madin-Darby Canine Kidney (MDCK – ATCC/CCL-34) cultivated in Eagle's minimum essential medium (EMEM - Gibco Laboratories, USA; SOUZA JÚNIOR *et al.*, 2010).

The epsilon toxin was standardized *in vitro* to the test level L+/50. Titration of the test sera was performed in 96 wells plates. Four repetitions of two-fold serial dilutions from 1:2.5 to 1:320 were prepared to a final volume of 50 µL/well. Another 50 µL was then added to each well containing standardized toxin at the test level L+/50. The plates were homogenized, re-incubated, and cellular suspensions (4.8×10^4 cells/50 µL) were added. Positive control wells contained 50 µL of standardized toxin, 50 µL of EMEM, and 50 µL of cells. Negative control wells had 100 µL of EMEM and 50 µL of cells. Serum control wells had 50 µL of the test serum, 50 µL of EMEM, and 50 µL of cells. The plate was incubated at 37°C in a humid chamber (Sheldon Manufacturing Inc – Oregon, USA) with a controlled atmosphere (5% CO₂ and 95% O₂) for 48 hours. The supernatant was removed after this period. Staining was

done with 100 µL of crystal violet solution (0.1% in alcohol) to observe the cytopathic effect (CPE) (SOUZA JÚNIOR *et al.*, 2010). Titers of the test sera were calculated as the highest dilution, resulting in 70% destruction of the cell monolayer (KNIGHT *et al.*, 1990). Retrotitration with standard antitoxin was used to check the standardization of the toxin (SALVARANI *et al.*, 2010). Results of *in vivo* and *in vitro* SN were analyzed and compared with a parametrical correlation using Pearson's correlation (SAMPAIO, 1998).

The results of *in vivo* and *in vitro* SN are shown in Table 1.

Titers obtained by *in vitro* SN were within intervals of the *in vivo* SN titers. The correlation between the results was 99.73% ($p < 0.05$), which was superior to the 91% previously demonstrated by KNIGHT *et al.* (1990). *In vitro* repeatability was 99.97%. Similar results were also previously reported by SALVARANI *et al.* (2010) and LIMA *et al.* (2011), which reported a correlation greater than 98% for alpha toxin of *Clostridium septicum* and for alpha toxin of *Clostridium novyi* type B, respectively. The viability of substituting the animal bioassay with *in vitro* models is thus proved.

Cell culture has a number of advantages over the animal bioassays. These include a significant decrease in the number of animals utilized, greater sensitivity and lower variation among individual responses. Additionally, an *in vitro* model that indicates cytotoxicity allows for the observation and study of the biological activity of the epsilon toxin, which is not attainable through other *in vitro* techniques such as ELISA and agar gel immunodiffusion.

This study demonstrates that detection of anti-epsilon-toxin by *in vitro* SN in MDCK cells is a viable option to replace the animal bioassays that are typically utilized in potency tests for clostridial vaccines containing epsilon toxoid.

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) and Instituto Nacional de Ciência e Tecnologia – Informação Genética Sanitária da Pecuária Brasileira. FCFL was supported by fellowship from CNPq (Brasília, Brazil).

Table 1. Titers (IU/mL) of anti-epsilon-toxin in serum pool of vaccinated rabbits and standard serum obtained by seroneutralization in the mouse and MDCK cells.

Serum	Antibody titer (IU/mL)	
	Mouse	Cells
T1	12.0	11.3
T2	6.0	5.6
T3	4.0	3.8
T4	10.0	10.2
T5	90.0	90.4
T6	180.0	180.8
T7 (negative control)	ND	ND

ND: not detectable.

REFERENCES

- AZEVEDO, E.O.; LOBATO, F.C.F.; ABREU, V.L.V.; MAIA, J.D.; NASCIMENTO, R.A. Avaliação de vacinas contra *Clostridium perfringens* tipos C e D. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, v.50, p.239-242, 1998.
- BORRMANN, E.; SCHULZE, F.; CUSSLER, K.; HÄNEL, I.; DILLER, R. Development of a cell culture assay for the quantitative determination of vaccination-induced antibodies in rabbit sera against *Clostridium perfringens* epsilon toxin and *Clostridium novyi* alpha toxin. *Veterinary Microbiology*, v.114, p.41-50, 2006.
- BRITISH PHARMACOPEIA. *Veterinary Antisera and Veterinary Vaccines*. 3.ed. Sainte Ruffine: Maisonneuve S.A., 1998.
- KNIGHT, P.A.; QUEMINET, J.; BLANCHARD, J.H.; TILLERAY, J.H. *In vitro* tests for the measurement of clostridial toxins, toxoids and antisera. II. Titration of *Clostridium perfringens* toxins and antitoxins in cell culture. *Biologicals*, v.18, n.4, p.263-270, 1990.
- LIMA, C.G.R.D.; LOBATO, Z.I.P.; PIRES, P.S.; SILVA, R.O.S.; SALVARANI, F.M.; ASSIS, R.A.; LOBATO, F.C.F. Padronização de teste de potência *in vitro* para vacinas que contenham toxoide alfa de *Clostridium novyi* tipo B. *Revista Arquivos do Instituto Biológico*, v.78, n.4, p.507-512, 2011.
- LOBATO, F.C.F.; MORO, E.; UMEHARA, O.; ASSIS, R.A.; MARTINS, N.E.; GONÇALVES, L.C.B. Avaliação da resposta de antitoxinas beta e épsilon de *Clostridium perfringens* induzidas em bovinos e coelhos por seis vacinas comerciais no Brasil. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, v.52, n.4, p.313-318, 2000.
- LOBATO, F.C.F.; LIMA, C.G.R.D.; ASSIS, R.A.; PIRES, P.S.; SILVA, R.O.S.; SALVARANI, F.M.; CARMO, A.O.; CONTIGLI, C.; KALAPHOTAKIS, E. Potency against enterotoxemia of a recombinant *Clostridium perfringens* type D epsilon toxoid in ruminants. *Vaccine*, v.28, n.38, p.6125-6127, 2010.
- MAPA – MINISTÉRIO DA AGRICULTURA, PECUÁRIA E ABASTECIMENTO. Portaria nº 49, de 12 de maio de 1997. Diário Oficial da União. Brasília, 16 de maio de 1997. Seção 1.
- METZ, B.; HENDRIKSEN, C.F.; JISKOOT, W.; KERSTEN, G.F. Reduction of animal use in human vaccine quality control: opportunities and problems. *Vaccine*, v.20, n.19, p.2411-2430, 2002.
- SALVARANI, F.M.; LOBATO, Z.I.P.; ASSIS, R.A.; LIMA, C.G.R.D.; SILVA, R.O.S.; PIRES, P.S.P.; LOBATO, F.C.F. *In vitro* evaluation of the alpha toxoid from *Clostridium septicum*. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, v.62, n.4, p.778-783, 2010.
- SAMPAIO, I.B.M. *Estatística aplicada à experimentação animal*. Belo Horizonte: Fundação de Ensino e Pesquisa em Medicina Veterinária e Zootecnia, 1998.
- SOUZA JÚNIOR, M.F.; LOBATO, Z.I.P.; PIRES, P.S.; SILVA, R.O.S.; SALVARANI, F.M.; ASSIS, R.A.; LOBATO, F.C.F. Padronização da titulação da toxina épsilon de *Clostridium perfringens* tipo D em linhagem contínua de células como alternativa ao bioensaio animal. *Ciência Rural*, v.40, n.3, p.600-603, 2010.