UNIVERSIDADE FEDERAL DO PARANÁ

VANESSA CARLI BONES

CONTRIBUTION TO THE IMPLEMENTATION OF VALIDATED ALTERNATIVE METHODS FOR RABIES DIAGNOSIS

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Supervisor: Professor Dr. Carla Forte Maiolino Molento

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A Comissão Examinadora da Defesa da Tese intitulada **"CONTRIBUTION TO THE IMPLEMENTATION OF VALIDATED ALTERNATIVE METHODS FOR RABIES DIAGNOSIS"** apresentada pela Doutoranda **VANESSA CARLI BONES** declara ante os méritos demonstrados pela Candidata, e de acordo com o Art. 79 da Resolução nº 65/09–CEPE/UFPR, que considerou a candidata apta para receber o Título de Doutor em Ciências Veterinárias, na Área de Concentração em Ciências Veterinárias.

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Professora Dra. Carla Forte Maiolino Molento
Presidente/Orientadora

Professora Dra. Rita Leal Paixão
Membro

Professor Dr. Mário Antônio Sanches
Membro

Professora Dra. Maria Fernanda Torres
Membro

Professora Dra. Simone Tostes de Oliveira Stedile
Membro

To the animals.

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In the silence I cried for them
Endless tears for voiceless victims
I have never seen tears ending their suffering
So I decided to do,
A fearless, deep, persistent and comforting way of doing
Relieving the agony of those who do not speak
Yes, they beg for help through their eyes
A way of doing that, to some people, may seem small
To me it is relief
Relief to see, little by little, my mission accomplished
And to know that this is not only *my* mission.

Vanessa Carli Bones

ABSTRACT

Laboratory animals are frequently used in many countries, despite the existence of validated alternative methods (VAM). The objective of this thesis was to collaborate to the implementation of VAM for rabies diagnosis in Brazil, contributing to the reduction of harmful laboratory animal use. The thesis was organized in six chapters: Chapter I is an introduction; II presents alternatives to the use of laboratory animals in Brazil and current opportunities to the development and use of alternative methods; III describes perceived barriers to the adoption of alternatives to laboratory animal use for rabies diagnosis, focusing on current rabies diagnosis methods performed in Brazil and other countries and barriers associated with replacing mice; IV presents a cost comparative study of the Mouse Inoculation Test (MIT) and the Virus Isolation in Cell Culture (VICC) for rabies diagnosis in Brazil, aiming to compare the costs to perform both test; V describes a decision tree (DT) to assist the replacement of laboratory animals in Brazil using rabies diagnosis as a model, focusing on the development of a framework applied to the Brazilian scenario; and VI adresses final considerations regarding all chapters. Chapter II shows that the Brazilian government is putting forward important initiatives, as the approval of the Law 11,794, which regulates the breeding and use of laboratory animals in the country, and the creation of organizations such as the Brazilian Centre for the Validation of Alternative Methods (BraCVAM) and the National Network of Alternative Methods (RENAMA); these initiatives indicate that Brazil is improving in the field of laboratory animal welfare. Chapter III is a result of a survey involving 12 Brazilians and 43 non-Brazilian respondents that performed rabies diagnosis. Many laboratories continue to use mice for rabies diagnosis and this proportion appears to be especially high in Brazil, despite animal protection laws and technical guidelines that favor the use of alternatives; the most frequently reported constraints associated with the use of alternatives were lack of laboratory facilities, equipment and materials and lack of financial resources. For chapter IV, considering that 200 MIT tests are equivalent to 350 VICC tests in terms of facilities and staff hours needed per month, we calculated the average total cost per sample tested and the costs of implementation of laboratory structure and routine use for both tests. In this sense, one sample analyzed by MIT costs around 205.2% more than by VICC; MIT costs 74.4% and 406.3% more than VICC considering implementation and routine use per month, respectively. For chapter V, we addressed barriers that hinder the replacement of animals described in chapter III and organized suggestions in a DT framework. The DT seems to have high resolution potential, provides guidance to address each obstacle and leads to the implementation or development of VAM. Our results collaborate to the implementation of VAM for rabies diagnosis in Brazil, contributing to the reduction of harmful laboratory animal use. Besides, results may be applied in other scenarios and by any person interested in implementing alternatives to animal use.

Key words: Animal welfare. Barriers. Cell culture. Economic analysis. Ethics on the use of animals. Framework. Laboratory animals. Mouse inoculation test. Regulation. Replacement. Survey. Three Rs.

RESUMO

Animais de laboratório são frequentemente utilizados em vários países, apesar da existência de métodos alternativos validados (Validated Alternative Methods- VAM). O objetivo desta tese foi colaborar para a implantação de VAM para o diagnóstico da raiva no Brasil, contribuindo para a redução do uso prejudicial de animais de laboratório. A tese foi organizada em seis capítulos: o Capítulo I é uma introdução; o II apresenta alternativas ao uso de animais de laboratório no Brasil e oportunidades para o desenvolvimento e uso de métodos alternativos; o III descreve barreiras percebidas à adoção de alternativas ao uso de animais de laboratório para o diagnóstico da raiva, focando em métodos diagnósticos realizados atualmente no Brasil e em outros países e barreiras associadas à substituição de camundongos; o IV apresenta um estudo comparativo de custos do teste de inoculação viral em camundongos (Mouse Inoculation Test- MIT) e do isolamento viral em cultivo celular (Virus Isolation in Cell Culture- VICC) para o diagnóstico da raiva no Brasil, e objetiva comparar os custos para realização de ambos os testes; o V descreve uma árvore de decisão (Decision Tree- DT) para auxiliar a substituição de animais de laboratório no Brasil usando o diagnóstico da raiva como modelo, focando no desenvolvimento de uma estrutura aplicada ao cenário brasileiro; e o VI apresenta considerações finais relacionadas a todos os capítulos. O Capítulo II mostra que o governo brasileiro está criando iniciativas importantes, tais como a aprovação da Lei 11.794, que regulamenta a criação e o uso de animais de laboratório no país, e a criação de organizações como o Centro Brasileiro de Validação de Métodos Alternativos (Brazilian Centre for the Validation of Alternative Methods- BraCVAM) e a Rede Nacional de Métodos Alternativos (National Network of Alternative Methods- RENAMA); tais iniciativas indicam que o Brasil está avançando no campo do bem-estar de animais de laboratório. O Capítulo III é o resultado de uma pesquisa envolvendo 12 respondentes brasileiros e 43 não brasileiros que realizavam o diagnóstico da raiva. Muitos laboratórios continuam utilizando camundongos para o diagnóstico da raiva e esta proporção parece ser especialmente alta no Brasil, apesar de leis de proteção animal e diretrizes técnicas que favorecem o uso de alternativas; as barreiras associadas ao uso de alternativas citadas com maior frequência foram falta de estrutura laboratorial, equipamentos e materiais, e falta de recursos financeiros. Para o Capítulo IV, considerando que 200 testes utilizando MIT são equivalentes a 350 utilizando VICC em termos de estrutura e horas de trabalho dos funcionários necessárias por mês, calculamos o custo total médio por amostra testada, além do custo para a implantação da estrutura laboratorial e do uso rotineiro de ambos os testes. Neste sentido, uma amostra analisada pelo MIT custa em torno de 205,2% mais que pelo VICC; o MIT custa 74.4% e 406.3% mais que o VICC considerando implantação e uso rotineiro por mês, respectivamente. Para o Capítulo V, descrevemos barreiras que impedem a substituição de animais descritos no Capítulo III e organizamos sugestões em uma estrutura em DT. A DT parece ter um alto potencial de resolução de barreiras, provê orientação para abordar cada obstáculo e leva à implantação ou ao desenvolvimento de VAM. Nossos resultados colaboram para a implantação de VAM para o diagnóstico da raiva no Brasil, contribuindo para a redução do uso prejudicial de animais de laboratório. Também, os resultados podem ser aplicados em

outros cenários e por qualquer pessoa interessada em implantar alternativas ao uso de animais.

Palavras-chave: Análise econômica. Animais de laboratório. Barreiras. Bem-estar animal. Cultivo celular. Estrutura. Ética no uso de animais. Inoculação viral em camundongos. Pesquisa. Regulamentação. Substituição. Três Rs.

LIST OF FIGURES

(VAM) IMPLEMENTATION. SHAPES MEAN: ▭ - INTERMEDIATE NODES, ⬭ - DECISION NODES, ⬡ - FINAL NODES

LIST OF TABLES

LIST OF ACRONYMS AND ABREVIATIONS

3Rs	-	Replacement, Reduction and Refinement
A	-	Anti-alternative
ACUCs	-	Animal Care and Use Committees
Altweb	-	Alternatives to Animal Testing Web Site
ANVISA	-	Agência Nacional de Vigilância Sanitária
ATCC	-	American Type Culture Collection
ATCs	-	Average Total Cost per sample
AU	-	Animal Use
AUEC	-	Animal Use Ethics Committee
AVCs	-	Average Variable Cost per sample
B	-	Brazilians
BHK	-	Baby Hamster Kidney
BraCVAM	-	Centro Brasileiro de Validação de Métodos Alternativos
CAAT	-	Center for Alternatives to Animal Testing
CCAC	-	Conselho Canadense de Cuidados aos Animais
CEUAs	-	Comissões de Ética no Uso de Animais
CNPq	-	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CONCEA	-	Conselho Nacional de Controle da Experimentação Animal
DOU	-	Diário Oficial da União
DT	-	decision tree
EURL ECVAM	-	European Union Reference Laboratory for Alternatives to Animal Testing
FAT	-	Fluorescent Antibody Test
FC	-	Fixed Costs
FIOCRUZ	-	Fundação Oswaldo Cruz
FRAME	-	Fund for the Replacement of Animals in Medical Experiments
IBAMA	-	Brazilian Institute of Environment and Natural Renewable Resources
ICMBio	-	Chico Mendes Biodiversity Conservation Institute
IEC	-	International Electrotechnical Commission

IFD	-	Imunofluorescência Direta
INCQS	-	Instituto Nacional de Controle de Qualidade em Saúde
INCQS	-	Instituto Nacional de Controle de Qualidade em Saúde
INMETRO	-	Instituto Nacional de Metrologia, Normalização e Qualidade Industrial
Interniche	-	International Network for Humane Education
IP	-	Pasteur Institute of São Paulo
ISO	-	International Organization for Standardization
IVC	-	Isolamento Viral em Camundongos
IVCC	-	Isolamento Viral em Cultura de Células
LC	-	Laboratory Coordinator
LNBio	-	Laboratório Nacional de Biociências
max.	-	maximum
MB	-	Main Branch
MCTI	-	Ministério da Ciência, Tecnologia e Inovação
Min.	-	minimum
MIT	-	Mouse Inoculation Test
n	-	number of tests performed
N2A	-	Linhagem de células do neuroblastoma de camundongos (murine neuroblastoma cell line)
NB	-	non-Brazilians
NGOs	-	Non-Governmental Organizations
NICEATM	-	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
NORECOPA	-	Norwegian Consensus Platform for Replacement, Reduction and Refinement of Animal Experiments
OECD	-	The Organisation for Economic Co-operation and Development
OIE	-	World Organisation for Animal Health
P	-	Pro-alternative
PPE	-	Personal Protective Equipment
RDC	-	Collegiate Board Resolution
RENAMA	-	Rede Nacional de Métodos Alternativos
RENAMA	-	Rede Nacional de Métodos Alternativos (National Network of

		Alternative Methods)
TC	-	Total Cost
UFMG	-	Universidade Federal de Minas Gerais
UNIPAR	-	Universidade Paranaense
USJT	-	Universidade São Judas Tadeu
VAM	-	Validated alternative methods
VC	-	Variable Costs
VICC	-	Virus Isolation in Cell Culture
WHO	-	World Health Organisation
ZEBET	-	German Centre for the Documentation and Validation of Alternative Methods

LIST OF SYMBOLS

nº	-	número
%	-	percentagem (percentage)
i.e.	-	that is, from the latim id est
p	-	statistical measure that represents how much evidence there is to reject the most common explanation for the data set
US$	-	United States Dollars
R$	-	Brazilian Reais
®	-	registered trademark
n	-	number

INDEX

1 PRESENTATION

Laboratory animals are frequently used in Brazil and in other countries, despite the existence of validated alternative methods (VAM). For rabies diagnosis, for example, the Mice Inoculation Test (MIT) can be replaced by the Virus Isolation in Cell Culture (VICC). The present work refers to the assistance for replacement of implementation of alternatives to the use of laboratory animals in Brazil; barriers to the adoption of alternatives to laboratory animal use for rabies diagnosis in Brazil and in other countries; a cost comparative study of the MIT and the VICC for rabies diagnosis in Brazil; and a decision tree to assist the replacement of laboratory animals in Brazil using rabies diagnosis as a model. Such studies are presented separately in chapters II, III, IV and V of this thesis.

Chapter II describes current opportunities to the development and use of alternative methods in Brazil, including opportunities from the government and related organizations. Such chapter was published in Portuguese by Vanessa Carli Bones and Carla Forte Maiolino Molento as a review paper at the Brazilian scientific journal *Veterinária em Foco,* volume 10, number 1, from pages 103 to 112, in 2012.

Chapter III describes perceived barriers to the adoption of alternatives to laboratory animal use for rabies diagnosis, which presents current rabies diagnosis methods performed in Brazil and other countries, and barriers associated with replacing mice. This chapter was published by Vanessa Carli Bones, Heloísa C. Clemente, a former student from the Federal University of Paraná, Daniel M. Weary, from the Animal Welfare Program of the University of British Columbia, in Canada, and Carla Forte Maiolino Molento. Such paper was published at the scientific journal Alternatives to Laboratory Animals (ATLA), volume 42, issue 3, from pages 171 to 179, in June 2014. Besides this publication, Appendices II, III, IV, IX, X and XI refer to related texts published at conferences and newsletters. Such study was approved by the Behavioural Research Ethics Board of the University British Columbia (Annex I).

Chapter IV refers to a cost comparative study of MIT and VICC for rabies diagnosis in Brazil, regarding the cost per sample tested, as well as the tests implementation and their routine use in the laboratory. This chapter was submitted to publication at ATLA, in August 2014, and was written by Vanessa Carli Bones, Augusto Hauber Gameiro, from the Socioeconomic Analysis and Animal Science

Laboratory (LAE) of the University of São Paulo (USP) Pirassununga campus, Juliana Galera Castilho, from the Pasteur Institute of São Paulo, and Carla Forte Maiolino Molento. Besides this publication, Appendices V, VI, IX, X and XI refer to related texts published at conferences and newsletters, and Appendix XII show the sheets containing the data collected and used for the cost comparison study.

Chapter V addresses barriers that hinder the replacement of animals described in chapter III and suggestions are organized in a DT framework. This text was written by Vanessa Carli Bones and Carla Forte Maiolino Molento. Besides this chapter, Appendices VII, VIII, IX, X and XI refer to related documents published at different conferences and newsletters.

Besides the publications presented on Appendices II to XI, a grant application related to this thesis was approved under the title *Subsídios à Implantação de Alternativas Validadas para Substituir o Uso de Animais de Laboratório: o diagnóstico da raiva como modelo* (Appendix XIII), *Chamada 24/2012 - Programa Universal – Pesquisa Básica e Aplicada* of the funding agency *Fundação Araucária*, from the State of Paraná, in 2014.

2 ALTERNATIVAS AO USO DE ANIMAIS DE LABORATÓRIO NO BRASIL

RESUMO

Animais de laboratório são amplamente utilizados no Brasil e em outros países; porém, tal situação tem gerado intensas discussões. Um marco importante para o bem-estar de animais de laboratório foi a publicação do conceito dos 3Rs- Replacement, Reduction e Refinement, que significa Substituição de animais, Redução do número de animais e Refinamento dos procedimentos envolvendo animais; o primeiro R é preferível em relação aos demais pois representa a substituição de animais vivos por métodos alternativos. Em função da preocupação por parte da sociedade e da necessidade de avanços na área de bem-estar de animais de laboratório, o governo brasileiro tem mostrado iniciativas importantes, a exemplo da aprovação da Lei Arouca que regulamenta a criação e a utilização de animais para ensino, testes e pesquisa no país. O Brasil é importante em termos de números de animais de laboratório utilizados; em determinadas situações os procedimentos podem ser substituídos por métodos alternativos. Por exemplo, para o diagnóstico da raiva a inoculação intracerebral de material suspeito em camundongos pode ser substituída por cultivo celular com alto grau de confiança. Apesar da disponibilidade de métodos alternativos aceitos internacionalmente, tais recursos não são utilizados por muitos laboratórios brasileiros, portanto é importante que se entendam quais as barreiras que impedem a substituição de animais para que se possa diminuir o sofrimento animal envolvido. Assim, oportunidades para a utilização de alternativas existem, fato que colaborou para a criação do Centro Brasileiro de Validação de Métodos Alternativos (BraCVAM) e da Rede Nacional de Métodos Alternativos (RENAMA). Além da criação de leis específicas e órgãos como a RENAMA e o BraCVAM, também se observa a inclusão de discussões relacionadas ao bem-estar animal e métodos alternativos em eventos científicos brasileiros; tais iniciativas demonstram que o país está gradativamente avançando no campo do bem-estar de animais de laboratório.

Palavras-chave: Bem-estar de animais de laboratório. Ética. Métodos Alternativos. Regulamentação.

ALTERNATIVES TO THE USE OF LABORATORY ANIMALS IN BRAZIL

ABSTRACT

Laboratory animals are widely used in Brazil and in other countries; however, this situation has stimulated intense discussions. An important milestone for the welfare

of laboratory animals was the publication of the 3Rs concept- Replacement, Reduction e Refinement, which means Replacement of animals, Reduction of the numbers of animals and Refinement of procedures involving animals; the first R is preferable in comparison to the others since it represents the replacement of live animals for alternative methods. Given the concern shown by society and the need for improvements in terms of laboratory animal welfare, the Brazilian government is putting forward important initiatives, such as the approval of the Arouca Law, which regulates the breeding and use of animals in teaching, testing and research in the country. Brazil is important in terms of numbers of animals used in laboratories; in certain cases, the procedures may be replaced by alternative methods. For example, considering the diagnosis of rabies, the intracerebral inoculation of suspected samples in mice may be replaced by cell culture with high degree of reliability. Although internationally accepted alternative methods are available, such resources are not used by many Brazilian laboratories. For this reason, it is important to understand the constraints that hinder the replacement of animals to decrease animal suffering. Thus, there are opportunities to the use of alternative methods in Brazil, fact that collaborated to the creation of the Brazilian Centre for the Validation of Alternative Methods (BraCVAM) and the National Network of Alternative Methods (RENAMA). Besides the creation of specific laws and organizations such as the BraCVAM and RENAMA, the inclusion of discussions related to animal welfare and alternative methods in scientific events in Brazil is also observed; such facts show that the country is gradually improving in the field of laboratory animal welfare.

Key words: Alternative Methods. Ethics. Regulation. Welfare of laboratory animals.

2.1 INTRODUÇÃO

Animais de laboratório são amplamente utilizados em diversas áreas como ensino, pesquisa, produção de medicamentos e diagnóstico de doenças. Porém, tal utilização gera crescentes discussões éticas. Discussões mais profundas em nível internacional tiveram início na Inglaterra na década de 50, com a publicação do livro *The Principles of Humane Experimental Technique* (RUSSEL; BURCH, 1992), cuja primeira versão foi publicada em 1959. Tal publicação resultou no surgimento do que pode ser considerado uma referência internacional para a ciência, os chamados 3Rs: Substituição de animais, Redução do número de animais e Refinamento dos procedimentos envolvendo animais, do Inglês *Replacement*, *Reduction* e *Refinement*.

Em síntese, a Substituição significa a utilização de material não-senciente em vez de animais vivos; a Redução diz respeito à diminuição do número de animais usados para obter uma informação, por meio da diminuição da quantidade de amostras, da utilização de técnicas estatísticas adequadas e da uniformidade da amostra para diminuir sua variação; o Refinamento remete a qualquer redução da severidade de procedimentos prejudiciais aplicados aos animais, incluindo o planejamento detalhado do experimento e a escolha adequada das espécies animais que serão utilizadas (RUSSEL; BURCH, 1992). Alguns cientistas consideram alternativas como sendo os 3Rs, porém neste artigo apenas a substituição será incluída na terminologia “métodos alternativos”.

Assim como ocorre em diversos países, no Brasil verifica-se uma crescente preocupação de cientistas, da indústria e em especial da sociedade acerca da utilização de animais de laboratório. Tal preocupação se caracteriza por elementos como sofrimento animal envolvido durante os procedimentos; ilegalidade da utilização de animais no Brasil quando existirem alternativas, determinada pelas Leis Federais nº 9605 (BRASIL, 1998) e nº 11.794 (BRASIL, 2008); reconhecimento internacional da necessidade da aplicação do conceito dos 3Rs (RUSSEL; BURCH, 1992), especialmente dos métodos substitutivos; maior eficiência de métodos laboratoriais mais modernos; a possível dissonância cognitiva enfrentada por laboratoristas envolvidos com a utilização de animais e a necessidade de trabalhar de forma atualizada. O objetivo desta revisão é descrever a situação atual referente às oportunidades de desenvolvimento e utilização de métodos alternativos no Brasil.

2.2 ASPECTOS NORMATIVOS

A regulamentação do uso de animais de laboratório no Brasil e em outros países foi revisada recentemente (BONES *et al*., 2010). Tal publicação confirma a necessidade de proteção do bem-estar animal e a preocupação crescente da sociedade com o sofrimento dos animais. Além dos movimentos sociais atuantes em prol da proteção animal, destaca-se a legislação específica em cada um dos países pesquisados e o trabalho das Comissões de Ética no Uso de Animais (CEUAs) presentes em instituições de ensino e pesquisa. Tal trabalho e as normativas relacionadas podem servir como alicerce para o progresso do controle das atividades que utilizam animais para ensino e pesquisa no Brasil, em nível estadual e federal.

A Lei Federal brasileira n° 9.605 de 1998, ou Lei de Crimes Ambientais, trata do uso de animais em experimentação e determina penalização a quem realiza experiência dolorosa ou cruel em animal vivo ainda que para fins didáticos ou científicos, quando existirem recursos alternativos (BRASIL, 1998). A Lei Federal nº 11.794 (BRASIL, 2008), ou Lei Arouca, regulamenta o inciso VII do artigo 225 da Constituição Federal, o qual incumbe ao Poder Público “proteger a fauna e a flora, vedadas, na forma da lei, as práticas que coloquem em risco sua função ecológica, provoquem a extinção de espécies ou submetam os animais a crueldade”. Ela complementa a Lei n° 9.605 mencionando em seu artigo 2º que “...aplica-se aos animais das espécies classificadas como filo Chordata, subfilo Vertebrata, observada a legislação ambiental” e dispõe sobre a criação e a utilização de animais para atividades de ensino e pesquisa, a definição de penalidades às instituições e profissionais pelo emprego indevido das normas, cria o Conselho Nacional de Controle da Experimentação Animal (CONCEA), no âmbito do Ministério da Ciência, Tecnologia e Inovação (MCTI), bem como estabelece a criação de CEUAs nas instituições que pratiquem a experimentação; dentre as atribuições do CONCEA está o cumprimento das normas relativas à utilização humanitária de animais usados em ensino e pesquisa, o credenciamento de instituições para criação ou utilização de animais, o monitoramento e a avaliação da introdução de métodos alternativos que substituam a utilização de animais, bem como a manutenção de um cadastro

nacional das CEUAs institucionais, dos procedimentos de ensino e pesquisa realizados e dos pesquisadores que realizam tais procedimentos.

As CEUAs tem a função de julgar o uso de animais em experimentação. No Brasil, os primeiros relatos de CEUAs datam da década de 90 (CHAVES, 2000); trabalhos descrevendo o funcionamento de tais comissões começaram a surgir a partir do ano 2000, destacando-se as publicações da Universidade Paranaense-UNIPAR (CIFFONI *et al*., 2001), Universidade São Judas Tadeu-USJT (BARBOSA, 2005), Universidade Federal de Minas Gerais-UFMG (OLIVEIRA, 2008) e Universidade Federal do Paraná (SILLA *et al*., 2009). Tais trabalhos demonstram que as CEUAs podem colaborar para o processo de controle do uso de animais em ensino e pesquisa, porém elas variam quanto à forma de trabalho. Somente com coerência de atuação, disponibilização de informações e atuação em conformidade com a legislação brasileira (BRASIL, 2008) as CEUAs poderão promover um sólido avanço ético na utilização de animais de laboratório. Para aumentar tal coerência de atuação poderiam ser incluídas no cadastramento nacional das CEUAs, por exemplo, informações detalhadas relacionadas aos projetos submetidos pelos professores e pesquisadores às comissões, tais como objetivos do uso dos animais, local de realização dos projetos, número de projetos aprovados e reprovados pelas CEUAs, grupos taxonômicos e números de animais listados, período de manutenção dos animais, grau de invasividade dos procedimentos, origem e destino dos animais, tempo de utilização dos animais, justificativa para sua utilização e comprovação de que os responsáveis estão considerando os 3Rs (RUSSEL; BURCH, 1992) e trabalhando de acordo com a legislação brasileira vigente para diminuir o sofrimento dos animais.

A partir das normas brasileiras mais recentes pode-se esperar maior organização e transparência dos dados relativos ao uso de animais em experimentação no futuro. O controle oficial derivado da Lei Arouca (BRASIL, 2008) encontra-se em construção, portanto tais informações não estão disponíveis. Neste sentido, Silla *et al*. (2010) investigaram o uso de animais em pesquisa através do método de amostragem bibliográfica, a partir de periódicos científicos publicados no estado do Paraná em 2006. Os resultados mostram um total, estimado por um cálculo conservador, de 3.497.653 animais usados, dos quais 216.223 foram vertebrados. Sessenta e sete por cento dos procedimentos foram classificados entre os graus A e B de invasividade, segundo a classificação proposta pelo Conselho

Canadense de Cuidados aos Animais (CCAC, 2006); 571 peixes foram empregados em procedimentos classificados como E, que envolve alto grau de sofrimento. Taylor *et al*. (2008), com base em artigos científicos publicados internacionalmente, estimaram que foi usado 1,16 milhão de animais vertebrados no Brasil em 2005, correspondendo a 11º posição entre os países que mais utilizam animais de laboratório no mundo. Os resultados sugerem que o Brasil parece ser importante no contexto mundial do uso de animais de laboratório, tanto em termos totais quanto em termos de animais vertebrados.

2.3 EXISTEM OPÇÕES?

Em muitos casos os animais de laboratório utilizados por instituições brasileiras podem lançar mão do conceito dos 3Rs. O primeiro R, o da Substituição, constitui a opção mais satisfatória pois representa a troca de métodos que usam animais para outros que não os utilizem. Exemplos de métodos substitutivos incluem modelos animais feitos de plástico, adequados para fases iniciais de aprendizado, materiais audiovisuais, programas de computador, métodos bioquímicos e imunológicos de análise e testes em organismos menores substituindo o uso de mamíferos, tal como ocorre em testes de metabolismo que utilizam hepatócitos de embriões de frangos (ENGH; SMITH, 2001). Outro importante exemplo de método alternativo citado por Engh e Smith (2001) é o cultivo celular, que utiliza células ou porções de órgãos obtidos de animais ou seres humanos, mantidos em solução rica em nutrientes, utilizados para produção de hormônios e vacinas, desenvolvimento de medicamentos, testes de toxicidade, produção de anticorpos e diagnóstico de enfermidades.

Apesar da existência de métodos alternativos capazes de substituir os animais de laboratório de forma eficaz, o número de animais usados no Brasil é alto. Para estudar os obstáculos a tal substituição é importante utilizar uma situação real como modelo. Tomando o diagnóstico da raiva como exemplo, realiza-se em primeira instância a Imunofluorescência Direta (IFD), que detecta antígenos virais usando anticorpos fluorescentes antivirais específicos, e, em casos inconclusivos, a sua confirmação por meio da inoculação intracerebral em camundongos, também chamada de prova biológica ou Isolamento Viral em Camundongos (IVC), ou por

meio do Isolamento Viral em Cultura de Células (IVCC) (WHO, 2005; MS, 2008; OIE, 2011). É o caso da técnica de isolamento viral para o diagnóstico da raiva em cultivo de células das linhagens *Baby Hamster Kidney* (BHK-21) (RUDD *et al*., 1980) e neuroblastoma de camundongos (N2A) (RUDD; TRIMARCHI, 1987). Ambas são recomendadas (OIE, 2008; OIE, 2011), mas as células N2A são mais sensíveis às espécies de vírus que acometem os animais naturalmente, o chamado vírus de rua, sem nenhum grau de adaptação (RUDD; TRIMARCHI, 1987; WHO, 2005; MS, 2008) e são altamente sensíveis à infecção por Lyssavirus em geral (OIE, 2011). As células da linhagem N2A (RUDD; TRIMARCHI, 1987), identificadas na American Type Culture Collection (ATCC) como CCL 131, são utilizadas em muitos países para o diagnóstico da raiva, inclusive no Brasil. De acordo com a OIE (2011), métodos alternativos como as técnicas de IVCC para o diagnóstico da raiva apresentam bons resultados se comparados com os rotineiros testes de referência de IFD e IVC e são mais adequados em termos de bem-estar animal por evitar sofrimento desnecessário.

Apesar do exposto, a inoculação de camundongos parece ser amplamente utilizada no Brasil. Bones, Weary e Molento (2012a) desenvolveram um estudo online cujo objetivo foi descrever métodos diagnósticos para a raiva utilizados em diferentes países atualmente. Os resultados mostram que, de um total de 47 participantes que trabalhavam com diagnóstico da raiva, 50% dos respondentes em português utilizavam a inoculação em camundongos, comparados a 20% em se tratando de respondentes em inglês (Figura 1). As principais barreiras que impedem a utilização de métodos alternativos para o diagnóstico da raiva apontadas por respondentes em português foram: falta de recursos humanos e capacitação profissional; acomodação, hábito e falta de boa vontade das pessoas; falta de recursos financeiros; barreiras regulatórias e falta de incentivo do governo; barreiras cultural e ética; falta de estrutura dos laboratórios, equipamentos e materiais; falta de conhecimento e conscientização; importância dos fatores orgânicos para observação da doença; baixa sensibilidade ou falhas das técnicas *in vitro*; facilidade e baixo preço do IVC; bem como falta de tempo (BONES *et al*., 2012b). Tais barreiras percebidas pelos respondentes denotam falta de investimento e iniciativa institucionais, bem como resistência das pessoas envolvidas, sugerindo que há oportunidade para aumentar a adoção de alternativas, pois algumas barreiras percebidas parecem imaginárias e outras são reais, mas passíveis de solução.

Como os resultados apontam para a utilização de camundongos também por parte de um porcentual dos respondentes estrangeiros, entender as barreiras à adoção do IVCC pode facilitar mudanças no Brasil e em outros países.

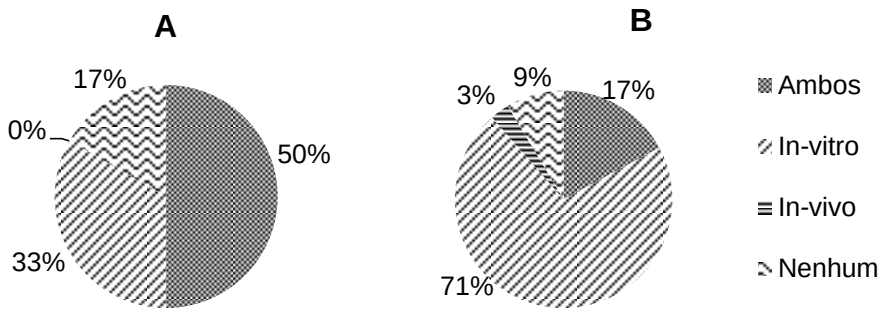

FIGURA 1 - MÉTODOS UTILIZADOS PARA O DIAGNÓSTICO DA RAIVA SEGUNDO ESTUDO ONLINE DESENVOLVIDO EM PARCERIA ENTRE A UNIVERSIDADE FEDERAL DO PARANÁ E A UNIVERSIDADE DA COLÚMBIA BRITÂNICA, CANADÁ. A- RESPOSTAS EM PORTUGUÊS. B- RESPOSTAS EM INGLÊS. (BONES *et al*., 2012a).

Também no ensino existem oportunidades claras para mudanças no que se refere à adoção de métodos alternativos ao uso de animais de laboratório. Deguchi *et al*. (2012) avaliaram as questões éticas envolvidas com a utilização de animais para propósitos educacionais no âmbito da Universidade Federal do Paraná. Para tal, foram entrevistados 101 estudantes e 20 professores de biologia, farmacologia, medicina e medicina veterinária. Metade dos estudantes não conhecia a legislação que regulamenta o uso de animais em educação e a maioria dos professores acredita que o aprendizado não pode ser obtido de forma adequada quando são utilizadas alternativas. Apenas 38.9% dos professores e 31.9% dos estudantes acreditavam na utilidade de tais métodos, sendo que os autores sugeriram ser necessária uma expansão da discussão referente às alternativas ao uso de animais no ambiente acadêmico (DEGUCHI *et al*., 2012). Talvez os profissionais que se formaram em tal ambiente acadêmico sintam necessidade de trabalhar de forma mais atualizada, beneficiando-se da utilização de alternativas, que são métodos mais modernos de ensino. Também pessoas que trabalham com animais nos laboratórios brasileiros talvez experimentem conflitos de ideias, crenças ou opiniões incompatíveis, estado comumente denominado dissonância cognitiva (DRAYCOTT; DABBS, 1998). Por um lado, os resultados mostram que tais pessoas reconhecem a

necessidade de evitar o sofrimento animal, por outro lado, talvez elas enfrentem dificuldades que impeçam ou dificultem mudanças e atualizações de métodos.

Segundo Bortolotti *et al*. (2008), as pessoas encontram dificuldades de quebrar paradigmas e alterar comportamentos, uma vez que as mudanças pressupõem algo novo, causando incertezas e resistência; porém, a resistência não constitui um obstáculo, mas sim uma oportunidade de transformação que pode ser útil desde que se descubram as suas causas. Os mesmos autores também destacam que a resistência pode chamar a atenção da sociedade para certos aspectos da mudança, de forma a minimizar as reações negativas associadas e promover formas de solucionar problemas por ela gerados.

No Brasil as propostas objetivando a criação de centros dedicados à implantação de alternativas ao uso de animais de laboratório começaram a surgir especialmente após a publicação da já referida Lei Arouca (BRASIL, 2008), que determina, entre outras provisões, o monitoramento e a avaliação quando da introdução de tais métodos. Em consonância com tais propostas, uma iniciativa importante em nosso país foi a criação do Centro Brasileiro de Validação de Métodos Alternativos (BraCVAM), vinculado ao Instituto Nacional de Controle de Qualidade em Saúde (INCQS), uma parceria entre a Fundação Oswaldo Cruz (FIOCRUZ) e a Agência Nacional de Vigilância Sanitária (ANVISA), cujo acordo de cooperação foi assinado em 2011 (ANVISA, 2011). De acordo com Presgrave *et al*. (2010), dentre os objetivos do BraCVAM estão a promoção e a divulgação de métodos alternativos, o treinamento e a educação sobre o conceito dos 3Rs (RUSSEL; BURCH, 1992) e a validação de métodos alternativos. Além disso, por meio da Portaria n° 491, de 3 de julho de 2012, o governo brasileiro criou a Rede Nacional de Métodos Alternativos (RENAMA), que terá duração de cinco anos contados a partir da data de sua publicação no Diário Oficial da União (DOU), podendo ser renovada por decisão do MCTI (MCTI, 2012). A RENAMA tem por objetivos estimular a implantação de alternativas ao uso de animais por meio do auxílio e do treinamento técnico nas metodologias necessárias; monitorar o desempenho dos laboratórios associados; promover a qualidade dos testes; incentivar a implementação do sistema de qualidade laboratorial; e promover o desenvolvimento, a validação e a certificação de novos métodos alternativos ao uso de animais, sendo o processo de validação das alternativas realizado no âmbito do BraCVAM (MCTI, 2012).

Segundo o MCTI, inicialmente a RENAMA será composta dos seguintes Laboratórios Centrais: Instituto Nacional de Metrologia, Normalização e Qualidade Industrial (INMETRO), o Instituto Nacional de Controle de Qualidade em Saúde (INCQS/FIOCRUZ) e o Laboratório Nacional de Biociências (LNBio). Gradativamente a Rede contará com a incorporação de outros laboratórios brasileiros com capacidade para contribuir para o seu desenvolvimento. Neste sentido, o MCTI, por meio do Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), publicou no dia 3 de setembro de 2012, no DOU, uma chamada pública com o objetivo de selecionar propostas para a estruturação da RENAMA (MCTI, 2012).

Em 2010 existiam 15 a 20 grupos de pesquisa trabalhando no desenvolvimento e na implementação de métodos alternativos ao uso de animais no Brasil, incluindo laboratórios oficiais, universidades, indústria e laboratórios privados (PRESGRAVE *et al*., 2010). Provavelmente este número cresceu nos últimos dois anos, mas a quantidade exata de grupos talvez seja conhecida após a seleção das propostas para estruturação da RENAMA e a consulta pública lançada pelo CONCEA em 2012. A “Consulta sobre a utilização de métodos alternativos ao uso de animais de experimentação” é uma espécie de formulário a ser preenchido pelos grupos que trabalham com alternativas, com o objetivo de mapear a situação brasileira atual relacionada ao desenvolvimento e implantação de tais recursos. É importante que os grupos trabalhem em sintonia e de acordo com a legislação brasileira e, segundo o MCTI (2012), a criação da RENAMA contribuirá para uma maior integração de trabalhos e estudos colaborativos relacionados aos métodos alternativos.

A importância do tema no contexto brasileiro também pode ser vista pela inclusão de discussões acerca do bem-estar animal de animais de laboratório e métodos alternativos em eventos científicos nacionais. Neste sentido, destaca-se a realização conjunta do I Congresso Latino-Americano de Métodos Alternativos ao Uso de animais no Ensino, Pesquisa e Indústria e da II Conferência Latino-Americana de Educação Humanitária e Alternativas, na cidade de Niterói-RJ (COLAMA, 2012), ocasião em que foram discutidas alternativas ao uso de animais de laboratório sendo desenvolvidas e utilizadas em diversos países, inclusive no Brasil, bem como os papéis do CONCEA, do BraCVAM e da RENAMA. Tais discussões, juntamente com a publicação de leis brasileiras objetivando a utilização discriminada de animais de laboratório e a criação de órgãos federais dedicados ao

estudo de métodos alternativos são resultados da exigência por parte da sociedade para um melhor tratamento dado aos animais e evidências de que o Brasil está gradativamente avançando no campo do bem-estar de animais de laboratório.

2.4 CONSIDERAÇÕES FINAIS

As discussões acerca do desenvolvimento e utilização de métodos alternativos ao uso de animais de laboratório no país estão se tornando cada vez mais frequentes, de acordo com a publicação de leis federais, as iniciativas de criação de órgãos especializados no desenvolvimento, validação e implantação de métodos alternativos e a realização de eventos científicos relacionados a tais alternativas. As oportunidades de mudança em termos de substituição dos animais existem em diversas áreas, como na pesquisa, no ensino, na indústria e também no diagnóstico de doenças. Apesar da disponibilidade de métodos alternativos eficazes, os laboratórios brasileiros utilizam animais em certos cenários. Portanto, para que se diminua o sofrimento animal envolvido é importante que sejam compreendidas as barreiras que impedem a adoção de tais alternativas. No caso específico do diagnóstico da raiva, as barreiras denotam falta de investimento e iniciativa institucionais, assim como resistência das pessoas envolvidas, havendo oportunidade para fomentar a adoção de alternativas. Conclui-se que a necessidade de implantar alternativas ao uso de animais de laboratório que correspondam à realidade e à legislação brasileira, que estejam de acordo com o princípio dos 3Rs e que primem pela redução do sofrimento animal pode ser considerada prioridade em nosso país.

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3 PERCEIVED BARRIERS TO THE ADOPTION OF ALTERNATIVES TO LABORATORY ANIMAL USE FOR RABIES DIAGNOSIS

ABSTRACT

The use of laboratory animals is still common practice, but some uses can be replaced by alternative methods, such as Virus Isolation in Cell Culture (VICC) instead of the Mouse Inoculation Test (MIT) for rabies diagnosis. The objective of this work was to describe current rabies diagnosis methods in Brazil and other countries, and the constraints associated with replacing this use of mice with alternative methods. Nine out of 12 Brazilian and 14 out of 43 non-Brazilian respondents reported that they currently used the MIT. Respondents in countries other than Brazil, male respondents, and those already employing *in vitro* methods for rabies diagnosis, expressed higher levels of support for the use of alternatives. The most frequently reported constraints associated with the use of alternatives were lack of laboratory facilities, equipment and materials (cited 17 times by respondents), and lack of financial resources (cited 15 times). The results indicate that many laboratories continue to use mice for rabies diagnosis. The proportion of laboratories that use mice appears to be especially high in Brazil, despite animal protection laws and technical guidelines that favour the use of alternatives. The barriers to the adoption of alternative methods identified in the current study provide a basis for facilitating changes in Brazil and elsewhere.

Key words: Animal welfare. Cell culture. Laboratory animals. Mouse inoculation test. Survey. Three Rs.

3.1 INTRODUCTION

The use of laboratory animals is common in many countries. In Brazil, the use of animals in research is governed by the Arouca Act (11.794/2008; BRASIL, 2008) and the Environmental Crimes Act (9.605/1998; BRASIL, 1998). The latter establishes penalties for performing painful procedures on laboratory animals, if alternative methods exist.

Rabies is a zoonosis present in more than 150 countries, killing approximately 55,000 people every year, and which is re-emerging as a serious public health problem in Africa, Asia and Latin America (WHO, 2013a; OIE, 2011). The disease does not cause easily identifiable clinical signs, making laboratory diagnosis essential. Several diagnostic methods have been published (ROBARDET *et al*., 2011); the Fluorescent Antibody Test (FAT) is considered to be the gold standard, with inconclusive tests confirmed through the Mouse Inoculation Test (MIT) or by Virus Isolation in Cell Culture (VICC; MS, 2008; OIE, 2011; WHO, 2013b), although it is recommended that the MIT be replaced by VICC whenever possible (OIE, 2011; WHO, 2013b; MAPA, 2009). The MIT involves the intracerebral inoculation of biological material from suspect individuals and uses 3–10 mice per sample (OIE, 2011; MS, 2008). The mice are then observed for 21 to 30 days: animals found dead after the fifth day post-inoculation are considered positive, and this is confirmed by the FAT. For VICC, *in vitro*- grown cells are inoculated with the biological material, subsequently treated with antibodies conjugated with fluorescein isothiocyanate and submitted to microscopic examination with ultraviolet light after 18 to 48 hours (MS, 2008). VICC is as sensitive as the MIT, produces results faster (OIE, 2011; MS, 2008; MAPA, 2009) and avoids animal use (OIE, 2011; MAPA, 2009).

The objective of this paper is to describe the extent of use of the MIT and alternative methods for rabies diagnosis in Brazil and other countries, and the perceived constraints associated with the adoption of these replacement methods.

3.2 MATERIAL AND METHODS

An online questionnaire was made available between September 2011 and August 2012, through the Your Views website (AHMAD *et al*., 2006). We recruited participants who were working on rabies diagnosis, via purposive and snowball sampling (PALYS, 2003). A list of potential participants was created based on our personal contacts, websites such as the Collaborating Centre for Control, Pathogenesis and Epidemiology of Rabies in Carnivores, the Global Alliance for Rabies Control, the World Health Organisation (WHO), the World Organisation for Animal Health (OIE), and the authorship of scientific papers related to rabies diagnosis. These individuals were sent invitations containing a short description of the study, a link, and information related to the group responsible for the research. This initial contact was followed by up to two reminders. To respond, the participant was asked to click on the link to access the survey (Table 1), in English or in Portuguese.

TABLE 1 - THE QUESTIONS AND ANSWERS SHOWN ON THE WEBPAGE FOR THE ONLINE SURVEY ON ALTERNATIVE METHODS FOR RABIES DIAGNOSIS.

Question/statement	Possible answers
“Age” (in years)	19-29, 30-39, 40-49, 50-59, more than 60
“Gender”	Female, male
“Education”	Secondary, College/university, masters, doctorate, other
“Country of residence”	-
“Which diagnostic tests for rabies are performed in your laboratory?”	*In vivo*, *in vitro*, both, neither
“Because”[a]	Open-ended
“What do you consider to be the constraints that prevent adoption of non-animal alternatives?”	Open-ended
“Are you performing a technique recommended by the WHO and the OIE?”	Yes, no
“Please describe which method you are using (e.g. the mouse inoculation test)”	Open-ended
“Does your laboratory diagnose rabies in:”	Humans, animals, both
“Do you work for a laboratory:”	Governmental, private, both
“Are you the person in your laboratory who decides what rabies diagnostic technique to use?”	Yes, no
“How long have you been working in the field of rabies	Less than 1, 1-5, 6-10, 11-20,

diagnostic testing?" (in years)	more than 21
"Does your institution have an animal care and use committee that oversees the use in testing?"	Yes, no
"Please briefly explain (in one sentence) how you define the 3Rs of ethical use of animals in science:"	Open-ended
"Mice can experience pain:"	Strongly agree, agree, disagree, strongly disagree, undecided

[a] Participants were asked to provide a reason for their choices, or select a reason provided by a previous participant that corresponded with their own thinking.

The participants were provided with a combination of multiple choice, Yes/No, Likert (ALEXANDRE *et al*., 2003) and open-ended text questions. The open-ended questions were analysed through collective subject discourse (LEFEVRE; LEFEVRE, 2010), a qualitative research technique that consists in grouping text responses with similar meanings. Reasons given for the questions "Which diagnostic tests for rabies are performed in your laboratory?" and "What do you consider to be the constraints that prevent adoption of non-animal alternatives?" were grouped in themes; themes for the first question were then classified as either 'pro-alternative' or 'anti-alternative' methods; because both open-ended questions (the method used and the constraints) are called reasons and were group in themes, but only the reasons for the use of a given method were classified as 'pro' or 'anti'.

Initially, the data were subjected to a descriptive analysis, where the participants were classified as 'pro-alternative' versus 'anti-alternative' methods based on themes classified as either 'pro' or 'anti', and according to the kind of rabies diagnostic test that they used. Themes classified as 'pro-alternative' versus 'anti-alternative' methods were also tabulated relative to the participant's country of residence (Brazilians or non-Brazilians), age, gender, education, power to make decisions, years of experience in the field, familiarity with the Three Rs, level of agreement with the statement that mice can feel pain, and the type of diagnostic test used. Contingencies were assessed by using the Chi-squared test. For themes 'pro-alternative' versus 'anti-alternative' relative to the type of diagnostic test used, contingencies were assessed in each group separately and also by comparing both groups of respondents.

3.3 RESULTS AND DISCUSSION

A total of 484 people were invited to participate, of which 129 lived in Brazil; 83 people agreed to participate, 16 in Brazil ('Brazilians') and 67 not in Brazil ('non-Brazilians'). Four Brazilians and 24 non-Brazilians were excluded from the analysis, because they answered only the first demographic questions or they did not use rabies diagnostic methods. The demographics of the participants are presented in Table 2. Of the non-Brazilian participants, 43 in total, six were in the USA, five in Canada, four in India, three in South Africa and Italy, and 22 in other countries in four continents.

TABLE 2 - THE NUMBERS OF RESPONDENTS ACCORDING TO LOCATION AND OTHER CRITERIA.

Question	Category	Brazilian	Non-Brazilian
Age (in years)	19–29	2	3
	30–39	3	10
	40–49	2	9
	50–59	5	14
	≥ 60	0	5
	No answer	0	2
Gender	F	7	12
	M	5	30
	No answer	0	1
Education	College/university	1	9
	Masters	3	10
	Doctorate	8	22
	Other	0	1
	No answer	0	1
Are you performing a technique recommended by the WHO and the OIE?	Yes	8	32
	No	1	5
	No answer	3	6
Does your laboratory diagnose rabies in:	Animals	6	15
	Humans	0	1
	Both	3	18
	No answer	3	9
Do you work for a laboratory:	Governmental	8	31
	Private	0	2
	Both	1	1
	No answer	3	9
Are you the person in your laboratory who decides what rabies diagnostic technique to use?	Yes	2	19
	No	7	15
	No answer	3	9
How long have you been working in the field of rabies	< 1	1	1

diagnostic testing? (in years)	1 to 5	1	9
	6 to 10	1	8
	11 to 20	2	7
	> 20	4	8
	No answer	3	10
Does your institution have an animal care and use committee that oversees the use in testing?	Yes	6	29
	No	3	4
	No answer	3	10

The sample consisted of 12 Brazilian and 43 non-Brazilian respondents.

In Brazil, the National Laboratory Network performs rabies diagnosis in 38 laboratories (MACHADO, 2011). At the international level, the WHO works in partnership with 12 collaborating centres for rabies research (WHO, 2012) and the OIE works with nine reference laboratories for rabies diagnosis (OIE, 2012). Four laboratories are connected with both the WHO and the OIE, so there are 17 reference laboratories worldwide. In this context, the participation of 12 Brazilian and 43 non-Brazilian respondents provides a meaningful sample.

Nine Brazilians and 14 non-Brazilians indicated that they used the MIT (Figure 2), including those that chose the option “both”. *In vitro* tests included the FAT (i.e. the ‘gold standard’ test), as well as VICC, flow cytometry, the Real-time Polymerase Chain Reaction, Direct Rapid Immunohistochemistry Test, Enzyme-linked Immunosorbent Assay, and antigen detection. The answer “both tests” referred to the use of the FAT, followed by the MIT to confirm the results; one Brazilian used “both” methods to perform quality control of rabies vaccines.

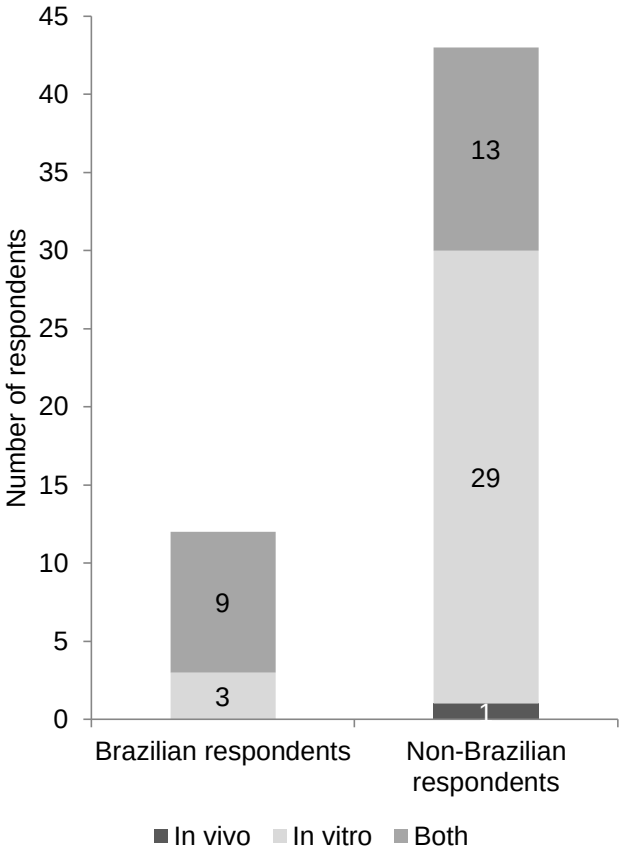


FIGURE 2 - THE TYPES OF RABIES DIAGNOSTIC TESTS PERFORMED BY 12 BRAZILIAN AND 43 NON-BRAZILIAN RESPONDENTS.

Table 3 shows the numbers of pro-alternative and anti-alternative respondents, relative to the tests they used. Two reasons provided by Brazilian participants and one by non-Brazilian participants were excluded from the analysis of the open-ended text responses, because they only repeated the name of the test used. Most of the Brazilians (i.e. seven) mentioned both 'pro-alternative' and 'anti-alternative' themes, while most of the non-Brazilians (i.e. 26) mentioned only 'pro-alternative' themes. As illustrated in Figure 2, most Brazilian respondents used both *in vitro* and *in vivo* methods, and most non-Brazilians used only *in vitro* methods.

TABLE 3 - THE NUMBERS OF RESPONDENTS RELATIVE TO THE STANCE (PRO-ALTERNATIVE VERSUS ANTI-ALTERNATIVE METHODS) AND THE TYPES OF RABIES DIAGNOSTIC TEST USED.

Method used[a]	Support for alternatives	Brazilian	Non-Brazilian
In vitro	Pro	1	26
	Anti	0	0
	Both[b]	0	3
Both methods	Pro	0	1
	Anti	2	0

	Both[b]	7	12
Total of respondents by kind of support	Pro	1	27
	Anti	2	0
	Both[b]	7	15
Total		10	42

[a]None of the respondents that used in vivo methods provided a text response to the question "Which diagnostic tests for rabies are performed in your laboratory?"; [b]refers to the themes both pro-alternative and anti-alternative methods.

The median number of 'pro-alternative' and 'anti-alternative' themes mentioned by each Brazilian respondent was 2 (min. = 0; max. = 6) and 1.5 (min. = 0; max. = 6), respectively. For non-Brazilians, the equivalent values were 3 (min. = 0; max. = 4) and 0 (min. = 0; max. = 2), respectively (Table 4). Of the 16 different themes, eight were pro-alternative and eight were anti-alternative. Of the eight 'pro-alternative' themes, six were mentioned by Brazilians and eight were mentioned by non-Brazilians. Of the eight 'anti-alternative' themes, all were mentioned by Brazilians, and four by non-Brazilians. Brazilians who used both methods, mostly cited themes that were anti-alternative (33 theme citations; $p = 0.005$); non-Brazilians who used exclusively *in vitro* methods, mostly cited themes that were pro-alternative (98 theme citations; $p < 0.0001$). Considering all the respondents, those employing *in vitro* methods were more likely to cite pro-alternative themes ($p < 0.0001$).

TABLE 4 - THE THEMES EXTRACTED FROM THE REASONS PROVIDED BY THE PARTICIPANTS IN RESPONSE TO THE QUESTION "WHICH DIAGNOSTIC TESTS FOR RABIES ARE PERFORMED IN YOUR LABORATORY?".

Theme of the answer, pro- (P) or anti- (A) alternative	Theme citations			
	Methods used			
	In vitro		Both methods	
	Brazilians[a]	Non-Brazilians[b]	Brazilians[a]	Non-Brazilians[b]
Reliability and high sensitivity of *in vitro* tests and possibility for replacing animals, P	1	30	7	12
MIT takes time and *in vitro* tests (e.g. VICC and FAT) give more-rapid results, P	2	38	0	0
Low cost of *in vitro* tests, P	0	23	0	0
Need for replacing animals for *in vitro* methods (ex. VICC), P	0	2	3	0
Replacement of animals for the VICC will happen soon, P	0	0	3	2

MIT is unnecessary and unjustifiable from scientific and ethical points of view, P	2	3	0	0
Lack of political will and technical awareness to replace the use of animals, P	1	0	2	1
Recommendation by WHO and OIE on animal welfare grounds, P	0	2	0	0
'Pro-alternative' themes — Subtotal[c]	6	98	15	15
MIT is necessary in some cases, A	0	3	6	11
MIT is reliable and sensitive, A	0	0	1	11
Need for specialised human resources and equipment to perform *in vitro*, A	0	2	7	1
Some tests are not accepted by the OIE or not licensed, A	0	1	5	0
High cost of *in vitro* tests, A	0	0	5	0
MIT is the preconized and the most used test, A	0	0	4	0
High possibility of mistakes when performing FAT, A	0	0	3	0
VICC takes time, A	0	0	2	0
'Anti-alternative' themes — Subtotal[c]	0	6	33	23

[a]The total number of theme citations from Brazilian respondents was 54; [b]the total number of theme citations from non-Brazilian respondents was 142; [c]this number is greater than the number of participants as some participants voiced more than one theme in their response.
The responses are classified relative to the country of residence (Brazilian versus non-Brazilian) and rabies diagnostic methods used by that participant.

One anti-alternative theme from Brazilian participants was that alternative methods for rabies diagnosis are expensive. In contrast, the most popular pro-alternative theme cited by non- Brazilian participants was that the alternative methods were less expensive.

For example, one non-Brazilian participant commented:

FAT and VICC are as sensitive as or more so than the MIT; as results are available within hours (FAT) to < 5 days (cell culture), rather than weeks, they are less expensive.

Thus, arguments about costs were used by both pro-alternative and anti-alternative respondents. This suggests that providing laboratories with more-accurate cost estimates of the various techniques may provide a powerful incentive to the adoption of alternative methods.

The location and gender of the respondents affected their support for alternative methods. Non-Brazilians and males were more likely to be pro-alternative methods than Brazilian and female respondents ($p < 0.05$). This effect of gender requires further study, as the literature has shown that females are more compassionate toward animals in general (KNIGHT; BARNETT, 2008), and more likely than men to oppose the use of animals in research (BROIDA *et al*., 1993; PIFER; SHIMIZU; PIFER, 1994). Age, education, power to make decisions, experience in the field, familiarity with the Three Rs, and agreement with the fact that mice can feel pain, all had no effect on support for the use of alternatives ($p > 0.05$).

Regardless of which methods were used, the participants described common constraints associated with the replacement of animals with alternative methods (Table 5). The most cited constraints were lack of laboratory facilities, equipment and materials, lack of financial resources, and lack of human labour and professional qualifications. All the respondents perceived that developing countries have more difficulties in investing in physical laboratory facilities and staff training to perform *in vitro* tests. For example, one comment was that:

Cell culture can be expensive, requires biosafety cabinets and requires fairly high levels of training. The FAT is subject to the skills of the reader for diagnosis and results may vary depending on the interpretation. Many labs, especially in Africa, do not have the necessary facilities for cell culture or the reagents and skills for the FAT, thus mouse inoculation is still widely used.

TABLE 5 - THEMES EXTRACTED FROM THE REASONS PROVIDED BY EACH PARTICIPANT IN RESPONSE TO THE QUESTION “WHAT DO YOU CONSIDER TO BE THE CONSTRAINTS THAT PREVENT THE ADOPTION OF NON-ANIMAL ALTERNATIVES?”.

Constraints	Brazilians	Non-Brazilians
Lack of laboratory facilities, equipment and/or materials	2	15
Lack of financial resources	3	12
Lack of human labour and professional qualification	5	8
Did not know/did not answer	3	6
Resistance: accommodation, habit, lack of goodwill	4	3
Regulatory barriers and lack of incentive by the government	3	3
There are no constraints	0	6
MIT is unavoidable	0	4
Lack of knowledge and awareness	2	1
Low sensitivity or flaws of in vitro techniques	1	2

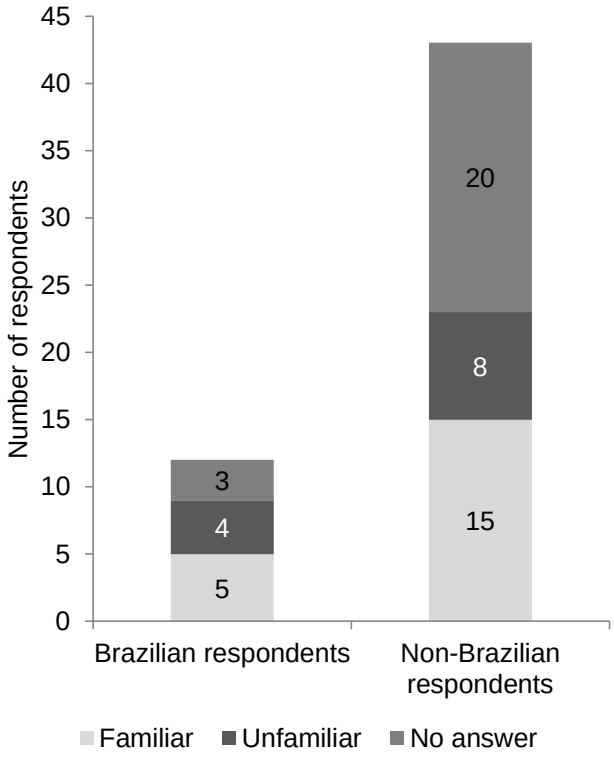
MIT is still extensively used	0	3
Difficulties to keep structure for cell culture	0	3
MIT is easier and less expensive	1	1
Poor moral decision-making	2	0
Importance of organic factors for disease observation	2	0
Structure to house animals already exists	0	2
MIT is more reliable	0	2
OIE and WHO are resistant to changes	0	1
Social agitation and poverty	0	1
Lack of time	1	0
Insecurity	0	1
Total	29	74

The responses are classified relative to the country of residence (Brazilian versus non-Brazilian).

Other barriers cited were: resistance to change; regulatory barriers; perceived low sensitivity; and flaws in the *in vitro* techniques. These results suggest the need for detailed follow-up interviews with participants, to better understand the factual basis of these perceptions.

Of the 55 Brazilian and non-Brazilian participants, 39 worked in government laboratories, and most of the participants (eight Brazilians and 32 non-Brazilians) performed tests as recommended by the WHO and the OIE, even though the types of diagnostic test varied. These results suggest that the WHO and the OIE could provide leadership in helping laboratories phase out the MIT.

Two Brazilian participants cited poor moral decision-making as a barrier to the adoption of alternatives - for example, not taking into account the detrimental effects of the MIT on the animals used. Animal Care and Use Committees (ACUCs) are typically in charge of providing ethical oversight for issues around animal use in institutions that use animals in research, teaching and testing. Thus, the work of the ACUCs would seem to be fundamental for laboratories employing the MIT in rabies diagnosis. However, only six of the 12 Brazilian respondents and 29 of the 43 non-Brazilian respondents reported that their institution maintained an ACUC. The ACUC is also responsible for training on, and application of, the Three Rs (RUSSELL; BURCH, 1992). The lack of functioning ACUCs may explain, in part, the lack of familiarity with the Three Rs among the participants in the current study (Figure 3).



Text responses to the question “Please briefly explain (in one sentence) how you define the Three Rs of ethical use of animals in science” were used to classify the respondents as either familiar or unfamiliar with the Three Rs concept.

FIGURE 3 - CLASSIFICATION OF 12 BRAZILIAN AND 43 NON-BRAZILIAN RESPONDENTS AS EITHER FAMILIAR OR UNFAMILIAR WITH THE THREE RS CONCEPT.

A case study on the barriers to the adoption of the Three Rs in the production, testing and evaluation of vaccines in 16 Canadian laboratories, suggested that increasing the harmonisation of the regulatory requirements would be helpful (LONG; GRIFFIN, 2012). A second study (FENWICK; DANIELSON; GRIFFIN, 2011) found that incentives and financial support could increase knowledge of the Three Rs and increase the welfare of the animals used in science. These findings are consistent with the findings of the current study, in that lack of knowledge and of incentives were highlighted as being barriers to the adoption of alternative methods for rabies diagnosis.

Interestingly, six non-Brazilian respondents who used *in vitro* methods, said that there were no barriers to the use of alternative methods. This variation in perspective suggests that a potentially useful approach might be to get people together in focus groups, allowing users to share perspectives and identify ways of addressing the perceived barriers. For example, one non-Brazilian participant mentioned that:

There are no constraints. A virology lab in any country can adapt [to using] in vitro techniques when given training.

The great majority of participants agreed that mice can feel pain. Only one Brazilian and one non-Brazilian strongly disagreed (Figure 4), and both of these participants were women. Overall, we found no relationship between support for the use of alternatives and the level of agreement with the claim that mice can feel pain. The opinions of people about animal sentience depend on several factors, such as their belief in the mental capabilities of animals, their affection or psychological attraction toward the animal species, and their concern for animal welfare (KNIGHT; BARNETT, 2008). However, the recognition of animal consciousness by formal scientific studies (LOW, 2012) should increasingly be taken into account. It is estimated that 58,339,972 laboratory animals were used in 2005, taking into account the available data from 179 countries (TAYLOR, 2008). Brazil seems important in this context, in terms of total numbers and in terms of vertebrate animals (SILLA; de OLIVEIRA; MOLENTO, 2010). For rabies diagnosis, in a laboratory that performs 200 MITs per month and inoculates eight mice per sample, the number of animals used would be around 19,200 per year. This estimation does not include animals that have to be replaced after nonspecific deaths from the trauma entailed from the intracerebral injection itself or from secondary infections following inoculation. The large number of animals used each year reinforces the importance of continued work to improve the adoption of alternative methods.

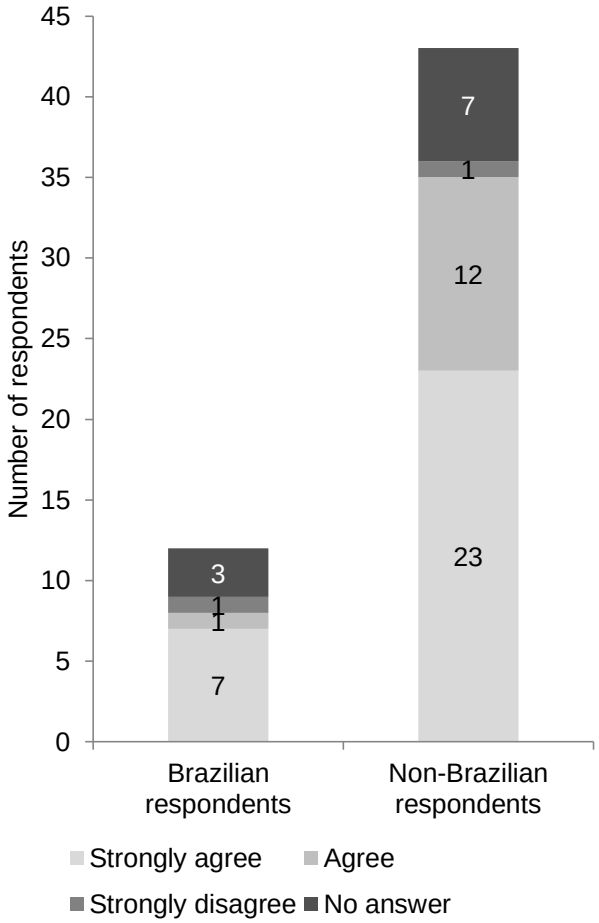


FIGURE 4 - THE RESPONSES OF 12 BRAZILIAN AND 43 NON-BRAZILIAN RESPONDENTS TO THE STATEMENT “MICE CAN EXPERIENCE PAIN”.

3.4 CONCLUSION

Despite the availability of non-animal alternatives, tens of thousands of animals are used for rabies diagnosis every year. Respondents in Brazil were more likely to use the *in vivo* MIT, than were respondents in other countries. The perceived high cost of *in vitro* methods was one of the reasons most frequently pointed out by Brazilian respondents for not adopting non-animal alternatives. Paradoxically, the low cost of *in vitro* methods was one of the reasons mentioned by most of the non-Brazilian respondents for employing these alternatives. Some respondents in countries other than Brazil also used the MIT. Thus, understanding the barriers to the adoption of alternatives may facilitate change in Brazil and elsewhere. Our results suggest that future engagement efforts should consider sharing best practices

among rabies diagnostic laboratories, including details on the full costs of the different diagnostic methods.

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4 COST COMPARATIVE STUDY OF THE MOUSE INOCULATION TEST (MIT) AND THE VIRUS ISOLATION IN CELL CULTURE (VICC) FOR RABIES DIAGNOSIS IN BRAZIL

ABSTRACT

Because the decision for using laboratory animals is frequently based on cost aspects, our objective was to compare the costs to perform Mouse Inoculation Test (MIT) and Virus Isolation in Cell Culture (VICC) for rabies diagnosis in Brazil. Based on the observation of laboratory routine at Pasteur Institute, São Paulo, we listed fixed (*FC*) and variable cost (*VC*) items necessary to perform both tests. Considering that 200 MIT tests are equivalent to 350 VICC tests in terms of facilities and staff hours needed per month, we calculated the average total cost per sample and the costs of 1) implementation of laboratory structure, and 2) routine use, for both tests. Regarding absolute values, the total cost was mainly influenced by *FC*, being 59.5% for MIT and 86.0% for VICC. Regarding percentage variation, one sample analyzed by MIT costs around 205.2% more than by VICC. MIT costs 74.4% and 406.3% more than VICC considering implementation and routine use per month, respectively. Our results contribute to the resolution of cost obstacles that hinder the replacement of animals for rabies diagnosis in Brazil. The method here demonstrated may be useful for cost comparisons on other situations of animal use when validated alternatives exist.

Key words: Animal Welfare. Economic Analysis. Laboratory Animals. Replacement.

4.1 INTRODUCTION

For rabies diagnosis it is recommended, as the first analysis, testing human or animal biological samples through the Fluorescent Antibody Test (FAT). Inconclusive results are confirmed through the Mouse Inoculation Test (MIT) or Virus Isolation in Cell Culture (VICC) (MS, 2008; OIE, 2011; WHO, 2013); even though it is recommended that MIT be replaced by VICC whenever possible (OIE, 2011; MAPA, 2009; WHO, 2013). Finally, MIT positive results should be confirmed through FAT (MS, 2008; OIE, 2011; WHO, 2013).

In Brazil the proportion of laboratories using mice for rabies diagnosis is high as compared to other countries (BONES et al., 2014). This situation is illegal according to the Brazilian federal legislation. The Federal Law 9605 (BRASIL, 1998), describes animal experimentation as a crime when alternative methods to replace animals exist. In the case of rabies diagnosis, alternatives to the use of animals, such as VICC, show satisfactory results and are more adequate on animal welfare grounds because they avoid unnecessary suffering (OIE, 2011). Besides, such methods present lower cost in comparison to the use of animals (MAPA, 2009; MS, 2008; OIE, 2011) once well implemented in the laboratory; in general, for rabies diagnosis, VICC costs approximately five times less than MIT (WEBSTER & CASEY, 1996). In spite of the information in the international literature, cost barriers to the adoption of *in vitro* methods for rabies diagnosis in Brazil were mentioned by Brazilians that worked in the field (BONES et al. 2014).

The word cost may be defined as the sum of expenses used to manufacture a product or provide services, including items such as consumed raw materials, employee salaries and payroll taxes, electricity and water, machine depreciation and maintenance, furniture, as well as other materials used in the productive process (WERNKE, 2005). Thus, the cost is composed by a set of technical (quantities of goods and services used) and economic coefficients (prices of such goods and services) involved in the productive process. Costs may be classified in fixed and variable, according to the production volume of a given unit, in a period of time (BERTÓ & BEULKE, 2005): fixed costs (*FC*) are those that do not depend on the production volume of the period, so, they do not change in the short-term basis, according to number of tests performed, for instance, machine maintenance and depreciation; variable costs (*VC*), on the other hand, are items that vary according to

the number of tests performed, so, for example, the higher the number of tests performed, the higher will be the consumption of reagents.

Because of ethical and legal impediments of animal use when alternatives exist, the plain justification to use animals frequently based on costs aspects without any detailed comparison, and also the inexistence of published information regarding the costs of MIT and VICC for rabies diagnosis, it seems important to compare the costs of both tests. The objective of our study was to compare the costs of MIT and VICC for rabies diagnosis in the Brazilian context, clarifying the judgment of the adequacy of claims regarding the impossibility for animal replacement due to cost reasons.

4.2 MATERIAL AND METHODS

The present study was based on the routine of the Pasteur Institute of São Paulo (IP), in November 2012, which allowed us to list materials, equipment and procedures needed for MIT and VICC performance. We considered the hypothetical situation of building model laboratories in Curitiba city, in the State of Paraná, South Brazil, which would imply in the following premises: i) values of items such as Basic Unit Cost of edifications (CBIC, 2012), electricity and water, as well as workforce, related to the State of Paraná; ii) official laboratories, which would imply on receiving conjugate and Challenge Virus Standard from IP for free, as is the normal procedure in all Brazilian Central Laboratories; and iii) monthly performance capacity of 200 tests by MIT and 350 by VICC, based on the average number of samples analyzed per month at IP between 2007 and 2011, and the dimensioned infrastructure. Other assumptions refer to rabies diagnosis being performed on samples originated from carnivores, which implies an MIT with 21 days-old mice weighing between 11 and 14 grams and up to 21 days post-inoculation observation. For VICC, the assumption was that results would be available within 18 to 48 hours followed the suspected sample isolation in cells (WHO, 2013).

We considered exclusively the total operational costs of the laboratory (BERTÓ & BEULKE, 2005) and those related to MIT and VICC. So, for example, the study did not include costs related to secretariat, administration, telephony and Internet; FAT test; safety items inherent to the laboratory, classified as biosecurity

level 2 (MS, 2008). Likewise, murine neuroblastoma cells, N2A line used for VICC were not included, since after the acquisition, if the laboratory possess adequate maintenance conditions, the cells can be multiplied and kept for an undetermined period of time (ATCC, 2012), therefore, characterizing a contemptible cost. The option not to consider such cost items was chosen by the fact that the values would be the same for both MIT and VICC, this way not influencing our study results.

The items necessary to perform MIT and VICC were organized in *FC* and *VC*, based on a cost comparison study which described two techniques to perform bovine viral diarrhea virus diagnosis (OLIVEIRA, 2013). The *FC* include the sum of monthly costs of the following groups of fixed items:

1- Depreciation of durable and semi-durable goods (equipment, varied utensils to support the tests and building). Depreciation was calculated because over time such goods loose value due to natural wear. To do so, for each item we divided the difference between the goods initial and residual value by their lifespan in months. The residual value refers to the goods value at the end of their lifespan, based on manufacturers and suppliers recommendations or, when this information was not available, on the authors' experience. Goods residual value varied from 0% over the initial value for glassware, plastic and utensils costing US$[1] 899.36 or below, to 10% for equipment and other utensils above US$ 899.36. The goods lifespan was established based on the Depreciation Taxes Table of the Brazilian Federal Revenue (RECEITA FEDERAL, 1998), on the manufacturers and suppliers recommendations, or on the IP staff experience.

2- Maintenance of durable and semi-durable goods (equipment, varied utensils to support the tests and building). Maintenance was calculated because over time such goods loose value due to natural wear and have to be repaired. Such value was established for each item based on the manufacturers and suppliers recommendations or on the authors experience, and varied between 0% over the initial value for glassware, plastic and other utensils, 10% for mechanical equipment, and 20% for electronic equipment.

3- Operational staff workforce that perform the tests and Personal Protective Equipment (PPE). For the staff workforce, besides salaries we included labor legal

[1] Values in Brazilian *Reais* (R$) were converted to United States Dollars (US$). On July 21st 2014, R$ 1.00 corresponded to US$ 0.4496807, according to the online currency converter of the Central Bank of Brazil, available at: <http://www4.bcb.gov.br/pec/conversao/conversao.asp> .

charges (BERTÓ; BEULKE, 2005), calculated as 60% of salary values for laboratory staff responsible for each test and one veterinarian technician responsible for MIT. For our study purpose the veterinarian is not the main responsible and not full time dedicated for MIT, so his/her workforce cost was determined using a ratio calculation dividing the total remuneration by the hours worked with MIT. Besides, we considered the laboratory staff those working for the government of State of Paraná, in the beginning of their carriers (PARANÁ, 2013), 40 hours per week.

4- Electricity, water and gases. To estimate the electricity and water consumption of MIT and VICC areas we considered the experience of one employee responsible for the PI general maintenance. To estimate nitrogen and carbon dioxide consumption we considered the suppliers recommendations or the IP staff experience.

5- Equipment licensing, including costs with equipment certification, calibration and preventive maintenance. With use, equipment loose calibration and have to go under maintenance and a quality certification process annually, this way meeting the requirements of the International Organization for Standardization (ISO)/ the International Electrotechnical Commission (IEC) 17.025 (ISO, 2005), in compliance with the Collegiate Board Resolution (*Resolução da Diretoria Colegiada*- RDC) 12 of the Brazilian Health Surveillance Agency (ANVISA, 2012).

The *VC* of both tests included reagents, varied materials to support the tests and PPE associated with the workforce, quantified based on the number of tests performed. To calculate *VC*, the unit value of each item was multiplied by the usage percentage of that item to test one sample. These values were summed and formed the Average Variable Cost per sample (*AVCs*), which was then multiplied by the number of samples analyzed in each test to obtain the total *VC*, a monthly value.

For the *VC* and *FC* calculation we quoted the market prices of all items considering they were brand new, through contact with manufacturers or suppliers via website, e-mail or telephone. The prices quotation was performed during the second semester of 2013. We did not include the goods cargo values, a commercialization modality known as Free on Board, and, in case of imported goods, the prices did not include import taxes considering that, in Brazil, goods used in laboratories are exempt from such taxes (RECEITA FEDERAL, 2014).

For MIT and VICC, *VC* and *FV* were summed to compose the Total Cost (*TC*), which subsequently were divided by the number of samples analyzed through

each test, leading us to the Average Total Cost per sample (*ATCs*), according to the following formulae:

$$TC = VC + FC \quad (1)$$

As, by definition:

$$AVCs = VC / n \quad (2)$$

We have:

$$VC = AVCs \, . \, n \quad (3)$$

Applying (3) in (1) we have:

$$TC = AVCs \, . \, n + FC \quad (4)$$

So, finally, the *ATCs* is given by:

$$ATCs = TC / n \quad (5)$$

Besides MIT and VICC *ATCs* calculation, we analyzed both under two perspectives regarding: 1) the implementation of the complete structure, considering that the laboratory does not yet perform rabies diagnosis; in this case, the *TC* was considered; 2) the routine use of the tests, considering that the *FC* are already available; in this case, only the *VC* were considered. Such analysis was based on items costs variation. The lower the variation, the higher the similarity in costs between both tests; also, a positive variation means that MIT costs more than VICC. Data tabulation and cost calculations were performed using Microsoft Excel® 2010 program.

4.3 RESULTS AND DISCUSSION

Considering both tests employed at maximum capacity, the *ATCs* of VICC was US$ 16.73 while that of MIT was US$ 51.06, showing that MIT was significantly less economic than VICC. Table 6 shows a comparative summary of costs related to the performance of rabies diagnosis tests through MIT and VICC; the table lines C and D refer to the results of the previously proposed formula (4) and (5).

TABLE 6 - SUMMARY OF COSTS NEEDED FOR THE PERFORMANCE OF MIT AND VICC FOR RABIES DIAGNOSIS AND PERCENTAGE OF VARIATION BETWEEN BOTH TESTS. DATA IS ORGANIZED IN FIXED COSTS (*FC*) AND VARIABLE COSTS (*VC*), NUMBER OF MAXIMUM SAMPLES TESTED (*n*), AVERAGE VARIABLE COST PER SAMPLE (*AVCs*), TOTAL COST (*TC*) AND AVERAGE TOTAL COST PER SAMPLE (*ATCs*). VALUES REFER TO THE SECOND SEMESTER OF 2013, CURITIBA-PR, BRAZIL.

Costs classification	VICC (US$)	MIT (US$)	Variation (% VICC)
A. Total *FC* (US$/month)	5,037.21	6,070.84	20.5
A.1. Depreciation of durable and semi-durable goods	766.57	849.20	10.8
A.2. Maintenance of durable and semi-durable goods	75.57	145.43	92.4
A.3. Operational workforce and PPE	3,287.55	3,956.50	20.4
A.4. Electricity, water and gases	770.93	1,061.24	37.6
A.5. Equipment licensing	136.59	58.47	-57.2
B. Total *VC* (US$/month)	817.74	4,140.38	406.3
B.1. Number of samples (*n*)	350	200	-42.8
B.2. *AVCs* (US$/sample)	2.34	20.70	786.1
C. *TC* (US$/month)	5,854.95	10,211.21	74.4
D. *ATCs* (US$/sample)	16.73	51.06	205.2

The only published work which compared the costs of VICC and MIT for rabies diagnosis mentions that, in general, VICC costs approximately five times less than MIT (WEBSTER; CASEY, 1996). Such conclusion was based on a proportion analysis of absolute values and is in agreement with our results when only the *VC* of both tests is considered. For other costs, our results show that VICC is 1.2 times more economic for *FC*, 1.7 for *TC* and 3.1 for *ATCs.*

If we compare both tests absolute values from Table 6, between total *FC* and *VC*, total *FC* accounts as the highest cost in both tests, being 86.0% for VICC and 59.5% for MIT. For VICC, *FC* was mainly influenced by the workforce, representing 56.1% of the *TC*. The high cost of workforce for VICC could be because the test requires skilled employees. Regarding animal use, a study proposed strategies that influence cost containment in animal research facilities, and claimed that direct labor personnel represents the largest cost item of an animal research facility, accounting for between 50% and 65% of the *TC* (NATIONAL RESEARCH COUNCIL, 2000). Our study shows a different scenario because the workforce was the second highest cost for MIT, representing 38.7% of the *TC*; the highest cost was the total *VC*. MIT

workforce absolute value was slightly higher than VICC's because it included one veterinarian technician responsible which is not mandatory for VICC.

The high cost of MIT workforce could be explained by the increasingly complex legislation, guidelines, and policies governing use of animals in research (NATIONAL RESEARCH COUNCIL, 2000), as well as by animal welfare demands and required training of personnel (GRUBER; HARTUNG, 2004). Legal and technical requirements also exist in Brazil (BRASIL, 2008; BRASIL, 1998). The Arouca Act (BRASIL, 2008), amongst other provisions, regulates the breeding and use of animals for teaching and research, defines penalties to institutions and professionals, creates the National Council for the Control of Animal Experimentation, and establishes the creation of Animal Care and Use Committees at institutions performing animal experimentation. Additionally, the Environment Crimes Act (BRASIL, 1998) explicitly forbids the use of animals when alternatives exist.

Total *VC* accounted for 14.0% of the VICC *TC* and 40.5% of the MIT *TC*, mainly due to the high costs of animals, feed and bedding, which together represent 91.1% of MIT total *VC*. Our results agree with published results in that food and bedding are likely to account for a high proportion of the supply costs, but they disagree with the information that supplies would account for only 11% of the budget of an animal facility (NATIONAL RESEARCH COUNCIL, 2000).

In terms of test implementation, MIT cost is 74.4% higher than VICC. For routine use, MIT cost is 406.3% higher than VICC. Both perspectives were influenced by MIT *VC* that was higher than VICC's.

Items classified as *VC* were the ones that most varied between tests overall costs, and in this sense, besides the positive variation of the total *VC* previously mentioned, MIT *AVCs* showed to be 786.1% higher than VICC *AVCs*. Such high variations were mainly influenced by the cost of the mice per se, as well as the bedding and feed used for maintaining the animals, which represent 76.0% and 15.1% of the MIT *AVCs* respectively. The *ATCs* also showed a high positive variation (205.2%), mainly influenced by *AVCs*, total *VC* and by the *n*, since MIT presents a lower capacity to analyze samples per unit input as compared to VICC.

Regarding the monthly capacity to analyze suspected samples, a laboratory performing MIT can analyze 42.8% less samples than if performing VICC. As a consequence, while VICC staff analyses 100 samples, MIT staff analyses only 57 samples. Such results show that a laboratory that uses alternative methods is more

efficient and economic, this way being more advantageous for society and the government in other ways beyond being more humane.

Besides the tests costs comparison based on *TC* and *VC*, we compared both tests regarding the variation of the other classified costs, all related to *FC*, as showed in Table 6. The only MIT item showing a lower cost in relation to VICC was the equipment licensing, being such cost 57.2% inferior for MIT as compared to VICC. Such fact is due to the need for quality certification of a higher number of equipment in the case of VICC. Costs presenting lower positive variations, meaning that they did not differ significantly between the tests, were depreciation of durable and semi-durable goods; operational workforce and PPE; electricity, water and gases; as well as the total *FC*. The remaining ones had higher positive variations, this way increasing the MIT cost considerably.

In this sense, the maintenance of durable and semi-durable goods varied 92.4%, mainly because we considered the use of an autoclave adequate to the high volume of waste originated from the MIT routine and also an acclimatized system with individually ventilated cages to house the animals; such items represent 48.9% and 34.3% of the test overall maintenance cost, respectively. In fact, it is recognized that an animal facility and the requirement to maintain reliable heating, ventilation, and air conditioning, electric systems, and sanitation and sterilization equipment dictate the need for constant maintenance (NATIONAL RESEARCH COUNCIL, 2000), this way contributing to increase the costs of an animal facility.

MIT would still cost more than VICC if some hypothetical situations were considered (Table 7). For example, we could consider that most Brazilian laboratories performing rabies diagnosis do not test such high numbers of samples as IP does. In this sense, the VICC sample analysis capacity was decreased in order to equal to the MIT maximum capacity. Besides, if mice were not housed in an acclimatized system, but in shelf racks and standard shoebox cages, what may be the reality in some Brazilian laboratories, the depreciation and maintenance costs of durable and semi-durable goods would be US$ 732.56 and US$ 95.48 respectively. Consequently, MIT *ATCs* would cost US$ 22.70 more if compared to VICC. Both simulations would result in an *ATCs* positive variation of 82.4%, meaning that MIT would still cost more than VICC.

TABLE 7 - SUMMARY OF COSTS NEEDED FOR THE PERFORMANCE OF MIT AND VICC FOR RABIES DIAGNOSIS AND PERCENTAGE OF VARIATION BETWEEN BOTH TESTS, CONSIDERING HYPOTHETICAL SITUATIONS IN BOLD. DATA IS ORGANIZED IN FIXED COSTS (*FC*) AND VARIABLE COSTS (*VC*), NUMBER OF MAXIMUM SAMPLES TESTED (*n*), AVERAGE VARIABLE COST PER SAMPLE (*AVCs*), TOTAL COST (*TC*) AND AVERAGE TOTAL COST PER SAMPLE (*ATCs*). VALUES REFER TO THE SECOND SEMESTER OF 2013, CURITIBA-PR, BRAZIL.

Costs classification	VICC (US$)	MIT (US$)	Variation (% VICC)
A. Total *FC* (US$/month)	5,037.21	5,904.25	17.2
A.1. Depreciation of durable and semi-durable goods	766.57	**732.56**	-4.4
A.2. Maintenance of durable and semi-durable goods	75.57	**95.48**	26.4
A.3. Operational workforce and PPE	3,287.55	3,956.50	20.4
A.4. Electricity, water and gases	770.93	1,061.24	37.7
A.5. Equipment licensing	136.59	58.47	-57.2
B. Total *VC* (US$/month)	467.28	4,140.38	786.0
B.1. Number of samples (*n*)	**200**	200	0
B.2. *AVCs* (US$/sample)	2.34	20.70	786.0
C. *TC* (US$/month)	5,504.49	10,044.62	82.5
D. *ATCs* (US$/sample)	27.52	50.22	82.5

The possibility of decreasing the cost of MIT by using shelf racks and standard shoebox cages instead of an acclimatized system, which ventilates cages individually, is not real. In fact, acclimatizing the whole room cost more than the individual cages (NATIONAL RESEARCH COUNCIL, 2000). For example, by not ventilating the mice cages but only the entire room, bedding will have to be changed more frequently to avoid odors and accumulation of irritable gases, and as a consequence, it will be necessary more workforce, as well as consumption of water, electricity and cleaning products.

Several publications mention that alternative methods are economic or more economic than the animal use. Examples of such publications include alternatives for diseases diagnosis, as for rabies (WEBSTER; CASEY, 1996), toxicity tests (VALADARES, 2006; KNIGHT, 2008), potency testing of vaccines (STOKES et al., 2011), teaching (van der VALK et al, 1999; SILVA, 2003; ABOUD et al., 2004; DEWHURST, 2004; WALSHAW, 2004; MAGALHÃES; ORTÊNCIO FILHO, 2006; FEIJÓ et al., 2008; TUDURY; POTIER, 2008; CUBO NETO, 2011; FOX et al, 2013;

RIBEIRO et al., 2013; SATHYANARAYANA, 2013), and research in general (KNIGHT, 2009). Such studies confirm our findings but none describe in details the method used to analyze the costs of the alternative resources, and only one presents the absolute costs for the development of the alternative method (RIBEIRO et al., 2013). The present method, here applied to rabies diagnosis, may be used to comparatively study the costs of the alternatives and the animal use in other scenarios, as the ones described in the previously mentioned publications and others.

4.4 CONCLUSION

Our results show that using live animal (MIT) costs more than the alternative method (VICC) for rabies diagnosis, considering the cost per sample analyzed, as well as for implementation and routine use of both tests in the laboratory. The proposed calculation method can contribute for the resolution of barriers to laboratory animal replacement, especially those related to costs, in other scenarios other than the rabies diagnosis. Besides, this is the first study that compare the costs of two important techniques used for rabies diagnosis and may be considered a strong argument for the implementation of VICC in Brazilian laboratories.

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5 A DECISION TREE TO ASSIST THE REPLACEMENT OF LABORATORY ANIMALS IN BRAZIL USING RABIES DIAGNOSIS AS A MODEL

ABSTRACT

Brazilian federal legislation makes the use of alternatives mandatory when validated methods to replace laboratory animals exist. The objective of this paper is to develop a decision tree (DT) framework to assist the replacement of laboratory animals in Brazil. Based on the rabies diagnosis scenario, we addressed barriers that hinder animal replacement, such as the ones regarding costs of alternative methods; the existence of qualified human resources in these methods; resistance by laboratory staff; incompatibilities between the Brazilian animal protection law and specific norms that allow or prescribe animal use; and the lack of government incentives. The DT presents a high resolution potential for the reported barriers to the replacement of laboratory animals in Brazil, provides guidance to address its main obstacles, and, step-by-step, leads to the implementation of validated alternative methods (VAM) or the VAM development in case such resources do not exist. The described DT seems suitable to be applied to scenarios of laboratory animal use where alternative methods exist, such as the rabies diagnosis, and can contribute to increase compliance with the 3Rs principles in science and with law requirements in Brazil.

Key words: Animal welfare. Barriers. Framework. Substitution. 3Rs.

5.1 INTRODUCTION

The need for replacing laboratory animals can be justified by the animal suffering caused during maintenance and experimental procedures, since they are sentient beings (LOW *et al*., 2012), by the higher costs of the animal use if compared to the use of alternatives (BONES *et al*., 2014a), and in Brazil by the fact that the Animal Protection Law (BRASIL, 1998) forbids the use of laboratory animals when alternative methods exist. This law is in agreement with the European Directive 2010/63/EU (EUROPEAN COMMISSION, 2010), which states that the use of animals for scientific or educational purposes should only be considered when a non-animal alternative is unavailable. The Directive represents an important step towards achieving the final goal of full replacement of procedures on live animals for scientific and educational purposes (EUROPEAN COMMISSION, 2010).

The full replacement of laboratory animals should be the final goal also in Brazil; nevertheless, animals are commonly used in some scenarios. The proportion of use of mice for rabies diagnosis, for example, is higher in Brazil (75%) compared to other countries (32%), considering answers from 55 respondents around the world (BONES *et al*., 2014b). The most frequent barriers cited by the respondents for the replacement of animals were lack of structure, equipment and materials in the laboratories; lack of financial resources; lack of human resources and professional qualification; resistance to change; as well as regulatory obstacles and lack of incentive by the government.

The Replacement of laboratory animals, the Reduction of animals and the Refinement of procedures involving animals are known by 3Rs Principles (RUSSEL; BURCH, 1992). These principles are recognized as essential to implement good practices involving animals in science (KNIGHT, 2009). Strategies that can increase compliance with the 3Rs principles include reproducibility and transfer of alternative methods (GRUBER; HARTUNG, 2004; DE BOO; KNIGHT, 2008) among users, through guidance how to properly search the databases (GRUBER; HARTUNG, 2004) and greater description and standardization of scientific papers methodologies (DE BOO; KNIGHT, 2008). Besides, a more severe obedience of animal welfare laws that require consideration and use of alternatives should become a prerequisite for financial support of researches, Animal Use Ethics Committee (AUEC) approval and publication of results; such measures would require education and cooperation of

funding agencies, AUECs and journal editors, about the potential of alternatives (KNIGHT, 2008). The objective of this paper is to develop a decision tree (DT) framework to assist the replacement of laboratory animals in Brazil.

5.2 MATERIAL AND METHODS

A thinking process which results in a choice among alternatives courses of action is known as decision making (TAGHAVIFARD; DAMGHANI; MOGHADDAM, 2009; TAYLOR, 2013). Choice requires that the implications of various courses of action be visualized and compared (TAGHAVIFARD; DAMGHANI; MOGHADDAM, 2009). In this sense, to facilitate decision making in the scenario of potential replacement of laboratory animals, we organized potential barriers and solutions in a decision tree (DT) framework. Based on computing sciences concepts (SHAH HAMZEI; MULVANEY, 1999), a DT is a structure often depicted in a top-down manner that comprises a finite number of nodes containing information, connected by lines.

The proposed DT offers a step-by-step procedure for overcoming barriers that may hinder the replacement of laboratory animals, based on one of our previous study focusing on the rabies diagnosis scenario (BONES *et al*., 2014b). Although the DT was based on a specific scenario, it was created considering potential application for other scenarios of laboratory animal use in Brazil to which alternative methods exist. Also, it is intended to be used by any person interested in replacing laboratory animals. People that might have interest to use the DT include laboratory directors and staff from public and private sectors that use animals in their work routine, researchers, as well as organized society members, such as members of animal protection organizations. The DT might also prove to be useful for people aiming to consult the existence of alternatives before submitting a project to an AUEC evaluation, since these Committees should only consider the approval of studies for which alternative methods do not exist.

The DT was created using the open-sourced software Dia Portable®. The framework was composed by intermediate, decision, recommendation and final nodes. Nodes were then connected using lines. To facilitate the framework visualization, the mentioned categories of information were shaped differently. The

Branches that derive from the Main Branch are presented from the most to the least cited barriers mentioned by people working with rabies diagnosis (BONES *et al*., 2014b); but such sequence should not be understood as mandatory. Following the framework development we then calculated the frequency and percentage of the barriers mentioned by people working with rabies diagnosis (BONES *et al*., 2014b) that the DT addresses.

5.3 RESULTS AND DISCUSSION

The DT starts with the question “Does Validated Alternative Method (VAM) exist?” (Figure 5 A and B). Searching for alternative methods to replace laboratory animals can be performed through specific databases such as the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM), National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), Fund for the Replacement of Animals in Medical Experiments (FRAME), Center for Alternatives to Animal Testing (CAAT), Alternatives to Animal Testing Web Site (Altweb), International Network for Humane Education (Interniche), Norwegian Consensus Platform for Replacement, Reduction and Refinement of Animal Experiments (NORECOPA), German Centre for the Documentation and Validation of Alternative Methods (ZEBET), The Organisation for Economic Co-operation and Development (OECD), and scientific publication databases such as ScienceDirect, Scopus, Pubmed and ProQuest; the last one contains several databases. Besides looking for alternative methods, scientists interested in replacing laboratory animals can also watch demonstrations on how to search for alternatives through courses, workshops or symposia aiming to increase scientific literacy (HART; WOOD; WENG, 2005).

The abilities to search for the 3Rs are limited according to the analysis of questionnaires applied to Animal Welfare Officers in Netherlands, which aimed to understand how such professionals obtain and use information related to the 3Rs in their laboratory daily work with animals (VAN LUIJK *et al*., 2013). The same limitation has been reported by researchers that use laboratory animals in the United States (USDA-APHIS-AC, 2000) and it seems to be the case in Brazil. To deal with such limitation in a long-term basis, it is important to formally implement education

programs and courses dealing with alternative methods (GRUBER; HARTUNG, 2004), bioethics and animal welfare (DEGUCHI; MOLENTO; SOUZA, 2012) in which animal handling is allowed.

If a VAM does not exist, it is necessary to plan its development, which depends on obtaining financial resources. The development of alternatives in areas of need, such as cosmetics, chemicals, chronic toxicity, neurotoxicity, immunotoxicity, should be encouraged by funding and awards (GRUBER; HARTUNG, 2004). For example, projects could be submitted to public research funding agencies or to business companies interested in investing in animal free products. While a VAM is being developed, it is necessary to submit projects involving laboratory animal use to AUECs (BRASIL, 2008). To increase consideration and use of the 3Rs principles (RUSSEL; BURCH, 1992), AUECs should require that project proponents search for alternative methods before planning a given animal use in research and present a document showing which databases were searched, which keywords were used and literary citations found (SHAPIRO, 1999). In Brazil, such requirement is established by the Brazilian Guideline for the Care and Use of Animals for Scientific and Teaching Purposes (CONCEA, 2013). However, when filling in the Standardized Form the Request of Authorization for the Use of Animals in Teaching and or Research (CONCEA, 2012), a project proponent does not have to present the results of a literature search for alternatives. The justification for using animals should be given to ethical review boards on a regular basis, this way making the scientist question his or her approach and the real need for the animal experiment (GRUBER; HARTUNG, 2004).

If a VAM exists, the next question is if the Laboratory Coordinator (LC) knows about the method. If not, it is necessary to expose the VAM to him or her and then to know if he or she is motivated to change. If no, due to resistance, lack of interest, oppression, and lack of financial or decisive autonomy, then knowledge about the Animal Protection Law (BRASIL, 1998) should be questioned. If the Law is not known, it is necessary to expose it to the LC. If, after exposing the law, the LC is still

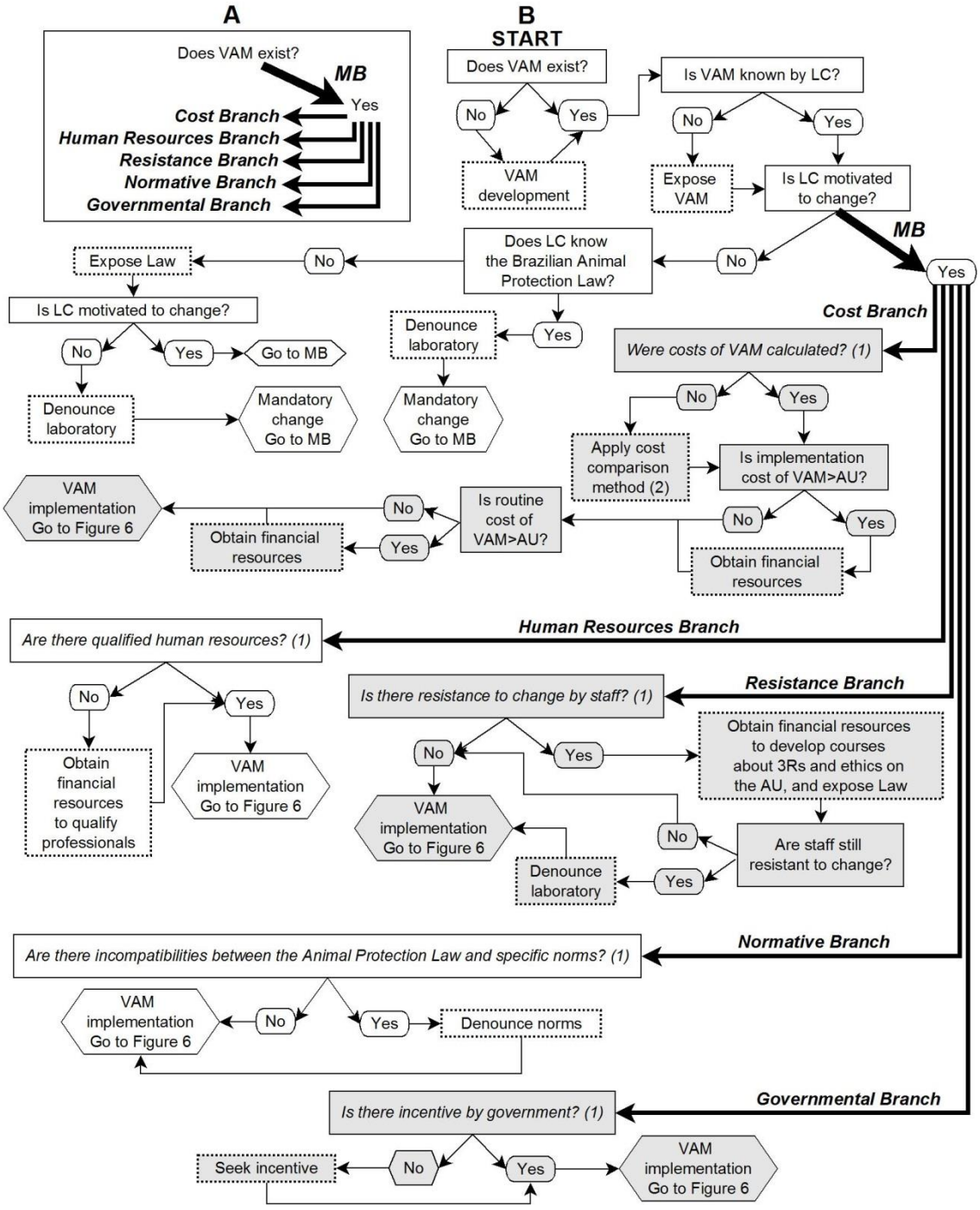

FIGURE 5 - A. OVERALL STRUCTURE OF THE DECISION TREE (DT) TO ASSIST THE REPLACEMENT OF LABORATORY ANIMALS IN BRAZIL. B. DETAILED DT TO ASSIST THE REPLACEMENT OF LABORATORY ANIMALS IN BRAZIL. SHAPES MEAN: ▭ - INTERMEDIATE NODES, ⬭ - DECISION NODES, ▭ - RECOMMENDATION NODES, ⬡ - FINAL NODES. VAM= VALIDATED ALTERNATIVE METHOD, LC= LABORATORY COORDINATOR, MB= MAIN BRANCH, AU= ANIMAL USE, 3RS= REPLACEMENT, REDUCTION AND REFINEMENT PRINCIPLES. (1) BARRIERS BASED ON BONES *et al*. (2014b); (2) COST COMPARISON METHOD BASED ON BONES *et al*. (2014a); LINES IN BOLD REFER TO THE MB AND OTHER BRANCHES.

not motivated to change, it is necessary to denounce the laboratory to appropriate official bodies and move to the Main Branch, since the change is mandatory. In case the LC knows the Animal Protection Law (BRASIL, 1998), and even then he or she is not motivated to change, it is also necessary to denounce the laboratory and move to the Main Branch. In order to denounce the use of laboratory animals when alternative methods exist, a citizen may register a law offence by an individual laboratory or a person. Alternatively, in the case of rabies diagnosis, when several laboratories might be acting against the law by using mice, the most recommended tools to seek animal replacement seem to be the Civil Public Action or the Popular Action. A Civil Public Action is generally proposed by the Public Ministry. However, its author may also be a legally established association amongst other institutions (LEVAI, 2004), such as legally established animal protection organizations. A Popular Action, on the other hand, may be proposed by any citizen, when the defendant is directly or indirectly a part of the public administration or a legal entity that administers public funds (BRASIL, 1965). There are several official bodies where it is possible to denounce the use of laboratory animals (Table 8), but the most straightforward ones, for their potential to inhibit and penalize the offender, include the Civilian Police and the Public Ministry. Once the complaint is registered, these official organizations should start a process involving investigation and gathering evidences of law offence regarding the situation reported. Alternatively, a person can gather evidences on his or her own or be represented by a lawyer in case of Civil Public Action or Popular Action proposals. It is also important to mention the media and the Non-Governmental Organizations (NGOs) pro-replacement of laboratory animals as unofficial bodies to report incompatibilities.

TABLE 8 - GOVERNMENTAL COMPETENCIES AND THEIR ADMINISTRATIVE AND JUDICIAL SCOPES TO DENOUNCE THE USE OF LABORATORY ANIMALS IN BRAZIL WHEN ALTERNATIVE METHODS EXIST.

Government Competencies	Administrative scope[a]	Judicial scope[b,c]
Municipal	Municipal Secretariat of the Environment	State Public Ministry represented by Prosecutor's Offices of Judicial Districts

State	State Secretariat of the Environment and related institutions	State Public Ministry, Civilian Police, Military Police, Environment Military Police
Federal	Brazilian Institute of Environment and Natural Renewable Resources (IBAMA), Chico Mendes Biodiversity Conservation Institute (ICMBio), Port Authorities, Navy Ministry	Federal Public Ministry, Federal Police

[a] Information based on the federal laws 6.938 (BRASIL, 1981) and 9605 (BRASIL, 1998);
[b] Information based on publications (LEVAI, 2004);
[c] Personal communication (ERBES[2], 2014).

If the LC is motivated to replace the laboratory animals for alternative methods, we are at the beginning of the Main Branch, formed by four Branches: Cost, Human Resources, Resistance, Normative and Governmental (Figure 6). Regarding the Cost Branch, it is first questioned if the costs of the VAM were calculated. If not, it is necessary to calculate them using a cost comparison method (BONES *et al*., 2014a), considering two perspectives: 1) the implementation of the complete structure, considering that the laboratory does not routinely perform a given method; for such, fixed and variable cost items, such as equipment and consumable products respectively, are included; 2) the routine use of the method, considering that the fixed costs items are already available; for such, only the variable costs are included. If the implementation of VAM costs more than the implementation of animal use it is necessary to obtain financial resources to structure the laboratory with fixed and variable cost items before questioning the cost of the routine use. For laboratories currently using animals, if implementation of VAM requires non-available items, it is also necessary to obtain financial resources. If the routine use costs more, it is necessary to obtain financial resources to buy variable cost items, before the VAM implementation.

For the Human Resources Branch, the first question is if there are qualified human resources available to implement the VAM. If not, it is necessary to obtain financial resources to qualify professionals. In order to qualify professionals on specific areas of knowledge, educational processes could be established, for

[2] ERBES, M. L. H. **Information regarding official bodies to denounce the use of animals when alternative methods exist**. Humaitá-RS, 2014. Personal communication.

example, through short-term courses performed by a multidisciplinary group of people specialized in alternative methods. Results of a study from the Japanese pharmaceutical area showed that many institutes lack education on proper conduct of animal experiments, including conduct related to the 3Rs, methods to evaluate and decrease distress and pain, and methods of euthanasia; so, further improvement seems necessary (OHNO, 2008). These results are in agreement with our perception that the need for a proper application of the 3Rs principles, especially regarding replacement, is urgent.

For the Resistance Branch, it is first questioned if there is resistance to change by the laboratory staff. If yes, it is necessary to obtain financial resources to educate people about the 3Rs (RUSSELL; BURCH, 1992) and ethics on the animal use. It is also relevant to make them aware of the Animal Protection Law (BRASIL, 1998). If after these education process staff is still resistant to change, then the laboratory should be denounced to appropriate official bodies. The resistance to change was confirmed by our research group; we invited people performing Mouse Inoculation Test (MIT) to implement the Virus Isolation in Cell Culture (VICC) for rabies diagnosis in Brazil. From 41 invited laboratories, only four (9.8%) showed interest, but none formally confirmed partnership. We also found resistance by laboratory staff in a previous study which surveyed people working with rabies diagnosis in different countries (BONES *et al*., 2014b); responses were associated with resistance including accommodation, habit and lack of goodwill.

The previously mentioned education process about the 3Rs and ethics on the animal use, if mandatory, may help decreasing the resistance to replace laboratory animals by staff. In this sense, a study was developed to assess the attitude of Portuguese participants in mandatory courses regarding the use of animals for the life sciences and the impact of the formal training in laboratory animal science on how participants see and apply the 3Rs (FRANCO; OLSSON, 2013). Based on self-administered questionnaires, the study results show that the course was effective in promoting awareness and increasing knowledge of the 3Rs, particularly regarding refinement, animal welfare, as well as consideration of animal ethics as being a relevant topic. However, participation in the course did not change perceptions on the current and future needs for animal use in research and most participants considered that even in the long-term, even partial replacement of animal experiments is unachievable. The little consideration of replacement is probably due to the focus of

the mentioned courses on reduction and refinement. Brazilian laboratory staff may also benefit from such courses; however, we emphasize the importance of focusing on replacement of laboratory animals. This will increase harmony between education of laboratory workers and the Brazilian Animal Protection Law (BRASIL, 1998) as well as the European Directive 2010/63/EU (EUROPEAN COMMISSION, 2010).

For the Normative Branch, it is first addressed if there are incompatibilities between the Brazilian Animal Protection Law (BRASIL, 1998) and specific norms that allow or demand the use of laboratory animals. If yes, it is necessary to denounce the specific norms to appropriate official bodies. For the rabies diagnosis scenario, Brazilian guidelines from the Ministry of Health and the Ministry of Agriculture, Livestock and Food Supply (MS, 2008; MAPA, 2009) describe the most common techniques: the Fluorescent Antibody Test (FAT) is considered the gold standard, with inconclusive tests confirmed through MIT or VICC, although it is recommended that the MIT be replaced by VICC whenever possible (MAPA, 2009); samples considered positive by MIT should be confirmed through FAT. Such national guidelines agree with international ones (OIE, 2011; WHO, 2013). Although such guidelines mention that VICC is sensitive, more economic and faster than MIT, at the same time they allow the use of animals when the laboratories do not have adequate facilities to perform VICC. This allowance for using animals stated by the national guidelines can be interpreted as illegal, since the federal animal protection law that forbids the use of animals when alternatives exist (BRASIL, 1998) is higher in the Brazilian law hierarchy. Although the replacement of animals by alternatives cannot occur fast in some scenarios, it is important that people that depend on such use start considering a gradual replacement and also that the federal government enforces the law and works toward the unification between the animal protection law (BRASIL, 1998) and specific norms.

For the Governmental Branch, if there is lack of incentive, one approach is to create opportunities to increase research related to alternatives. Official research funding agencies, stimulated by society, may offer specific grant applications for research related to alternatives to replace laboratory animal use. In this sense, the National Network of Alternative Methods (RENAMA), was created in Brazil in 2012 to stimulate the implementation of alternatives to the animal use through technical training of the necessary methodology; to monitor the performance of associated laboratories; to promote test quality; to support the implementation of the laboratorial

quality system; and to promote the development, validation and certification of new alternative methods (MCTI, 2012). In 2012 the government invited research groups in Brazil to submit projects related to the development or implementation of alternative methods and some were selected to become part of RENAMA. RENAMA is an example of government incentive, and its transformation into a perennial effort should be sought, thus maintaining the allocation of financial resources for the selection of projects focusing not only on the development of new alternative methods but also on the transfer and use of VAM. To achieve such degree of incentive by the government, public funds spent on animal tests required by regulators could be redirected into further development and implementation of alternatives (GRUBER; HARTUNG, 2004).

The situation of laboratory animal use and the possibility to use alternatives in some scenarios must be transparent to the general public, since transparency about the use of animals in science is the way to engender public support (CLARK, 2014; KIM, ORMANDY, WEARY, 2014). This is a pre-requisite for well based public movements to ask government permanent and increasing incentive for researchers interested in using alternatives. To seek more incentive, people could act by themselves, through petitions, by the election of politicians interested in working for the implementation of alternatives and prepared to represent the public locally or nationally, as well as by local committees dedicated to the animal protection in cities where they exist.

Following the VAM implementation in all Branches, if such method depends upon materials from animal sources, the beginning of the DT should be addressed again (Figure 6), in order to replace the animal use for the production of such materials. For rabies diagnosis through VICC, examples of materials used in Brazilian laboratories that still depend upon animals are antibodies conjugated with fluorescein isothiocyanate, Challenge Virus Standard, Normal Mice Brain and fetal bovine serum. Besides, VICC for rabies diagnosis should be preferably performed with Murine Neuroblastoma Cells, N2A line (OIE, 2011; WHO, 2013). When first multiplied in laboratory for commercial purposes, decades ago, N2A cells depended on the use of animals; today the cells can be kept for an undetermined period of time (ATCC, 2012), if the laboratory has the conditions to allow such long-term maintenance.

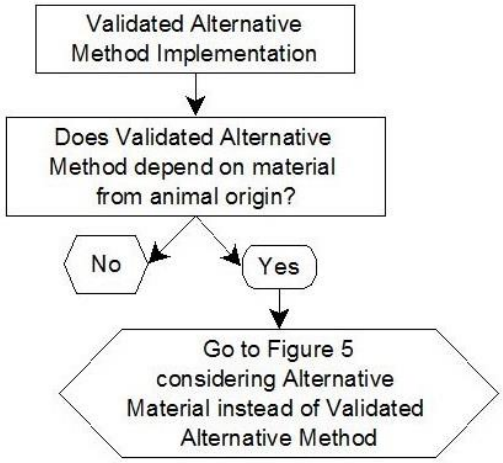

FIGURE 6 - DECISION FRAMEWORK TO ASSIST THE REPLACEMENT OF MATERIAL FROM ANIMAL ORIGIN IN THE CONTEXT OF VALIDATED ALTERNATIVE METHOD (VAM) IMPLEMENTATION. SHAPES MEAN: ▭ - INTERMEDIATE NODES, ⬭ - DECISION NODES, ⬡ - FINAL NODES.

Based on the previous analysis, it is possible to infer the DT potential to solve barriers, based on the rabies diagnosis scenario (BONES *et al*., 2014b), as presented in Table 9. So, considering the order proposed by Figure 6, the question node “Is VAM known by LC?” solves 10.4% of barriers mentioned by Brazilians (B) and 5.4% by non-Brazilians (NB). The Branch Cost solves 20.7% of barriers mentioned by B and 43.3% by NB; the Human Resources solves 24.1% of barriers mentioned by B and 13.6% by NB; the Resistance solves 17.3% of barriers mentioned by B and 16.1% by NB; the Normative solves none of the barriers mentioned by B and 1.4% by NB; and the Governmental solves 10.3% of barriers mentioned by B and 4% by NB. Then, for B respondents the Human Resources Branch has the highest potential to solve barriers and for NB respondents the Cost Branch seems to have the highest potential. However, other barriers presented might predominate or even different ones might be identified in other contexts, so the DT barrier solving potential depends on each laboratory specific situations.

TABLE 9 - QUESTION NODES OF THE DECISION TREE (DT) FRAMEWORK FOR ASSISTING THE REPLACEMENT OF LABORATORY ANIMALS IN BRAZIL ASSOCIATED WITH BARRIERS THAT PREVENT ADOPTION OF NON-ANIMAL ALTERNATIVES FOR RABIES DIAGNOSIS (BONES *et al*., 2014b); BARRIER CITATIONS ARE CLASSIFIED RELATIVE TO PARTICIPANT COUNTRY OF RESIDENCE.

Question nodes	Barrier	Barrier citations	
		Brazilians,	Non-Brazilians,

		n (%)	n (%)
Is VAM known by LC?	Low sensitivity or flaws of *in-vitro* techniques	1 (3.5)	2 (2.7)
	Importance of organic factors for disease observation	2 (6.9)	0 (0)
	MIT is more reliable	0 (0)	2 (2.7)
	Subtotal	**3 (10.4)**	**4 (5.4)**
Were the costs of VAM calculated?	Lack of structure of laboratories, equipment and or materials	2 (6.9)	15 (20.3)
	Lack of financial resources	3 (10.3)	12 (16.2)
	Difficulties to keep structure for cell culture	0 (0)	3 (4)
	MIT is easier and less expensive	1 (3.5)	1 (1.4)
	Social agitation and poverty	0 (0)	1 (1.4)
	Subtotal	**6 (20.7)**	**32 (43.3)**
Are there qualified human resources?	Lack of human labor and professional qualification	5 (17.2)	8 (10.8)
	Lack of knowledge and awareness	2 (6.9)	1 (1.4)
	Insecurity	0 (0)	1 (1.4)
	Subtotal	**7 (24.1)**	**10 (13.6)**
Is there resistance to change by staff?	Resistance: accommodation, habit, lack of goodwill	4 (13.8)	3 (4)
	MIT is unavoidable	0 (0)	4 (5.4)
	MIT is still extensively used	0 (0)	3 (4)
	Structure to house animals already exists	0 (0)	2 (2.7)
	Lack of time	1 (3.5)	0 (0)
	Subtotal	**5 (17.3)**	**12 (16.1)**
Are there incompatibilities between the animal protection law and specific norms?	OIE and WHO are resistant to changes	0 (0)	1 (1.4)
	Subtotal	**0 (0)**	**1 (1.4)**
Is there incentive by government?	Regulatory barriers and lack of incentive by the government	3 (10.3)	3 (4)
	Subtotal	**3 (10.3)**	**3 (4)**
	Did not know/ did not answer	3 (10.3)	6 (8.1)
	There are no constraints	0 (0)	6 (8.1)

	Poor moral decision making	2 (6.9)	0 (0)
	Total	**29 (100)**	**74 (100)**

Abbreviations mean: Validated Alternative Methods (VAM); Laboratory Coordinator (LC); Mouse Inoculation Test (MIT); World Organization for Animal Health (OIE); and World Health Organization (WHO).

By summing the five Branches barriers percentages (Table 9), it is possible to infer that the Main Branch of the DT solve most of the barriers hindering the replacement of laboratory animals cited by Bones and colaborators (2014b), being 72.4% mentioned by B and 78.4% by NB. Adding such percentages to the ones from the question node “Is VAM known by LC?” the DT directly solves 82.8% of barriers mentioned by B and 83.8% by NB. Such potential to solve barriers does not comprise the barriers “Did not know/ did not answer”, “There are no constraints” and “Poor moral decision making”; however, these are indirectly related to the DT. In this sense, poor decision making is related to all Branches and it constitutes one of the main motivations of the present study. Additionally, the lack of barrier identification by NB respondents might not be considered as a barrier per se. Thus, we expect that all known barriers that hinder the replacement of laboratory animals for rabies diagnosis (BONES *et al*., 2014b) may be dealt with by the proposed DT, characterizing a high resolution potential. We also believe that these results may be adapted to other scenarios of laboratory animal use.

Barriers for the adoption of alternative methods can also be identified in other areas of knowledge. For teaching, barriers include lack of knowledge about alternatives and opportunities to test them (DINIZ *et al*., 2006; MAGALHÃES; ORTÊNCIO FILHO, 2006); the belief that using live animals is more realistic than using alternatives, that many alternatives available on the market are not cheap and their quality does not always correspond to price, as well as lack of financial resources (RUKSENAS, 2005); resistance of some faculty members and concerns about their educational efficacy (DINIZ *et al*., 2006; MAGALHÃES; ORTÊNCIO FILHO, 2006; KNIGHT, 2007); little amount of discussion on alternatives in the academic environment, lack of awareness by undergraduate students regarding legislation regulating the use of laboratory animals in Brazil, lack of trust on the usefulness of alternative methods by lecturers and students and lack of institutional

guidance or support for the adoption of alternatives (DEGUCHI; MOLENTO; SOUZA, 2012).

Similarly, for basic research and research in general, identified barriers include lack of information and awareness about the 3Rs principles, lack of detailed description of published papers and databases allowing permanent updating on such studies methodologies, lack of facilities to perform *in vitro* studies and scientists reluctance to change work methods (GRUBER; HARTUNG, 2004). Also, for safety tests of new products, in some cases decision makers in regulatory bodies are unaware of new methodologies and are hesitant to adopt them (CURREN *et al*., 2014). This resistance may be represented by intellectual inertia, lack of time to learn new techniques and willingness to obtain research grants based on projects that depend on animals (GREEK; GREEK, 2004). The same authors mention that resistance to stop laboratory animal use can also be seen in the industry, since equipment and materials necessary to maintain animals such as cages, feed, and instruments are responsible for significant profits for specific industries. All mentioned barriers for replacing animals in teaching, research and tests of products seem comparable to the barriers for implementing alternative methods for rabies diagnosis (BONES *et al*., 2014b). Thus, the proposed DT may be useful to contribute to the replacement of laboratory animals in other areas where alternative methods exist.

Regardless of the barriers encountered in a laboratory that has the potential to replace animals by alternatives, it seems important to consider the strategy to approach the laboratory. When the demand for replacing animals is internally created by the staff, replacement will probably occur faster and more smoothly than if proposed by outsiders. In this sense, internal demand may be stimulated by increasing staff perception of the advantages of alternatives. An additional approach is to offer support to those laboratories that have potential to replace animals through a consulting service which adapts available information to the reality of each laboratory. Also, it is important that the Brazilian government recognizes the need for replacing animals and funds studies in alternatives, redirecting investments from research based on animals to that based on alternative methods . This movement is needed and may represent an increase in terms of law adherence by the government.

5.4 CONCLUSION

The described DT seems suitable to be applied to scenarios of laboratory animal use where alternative methods exist, such as the rabies diagnosis, our study model. By dealing with barriers that commonly hinder the replacement of laboratory animals, the proposed DT can contribute to increase compliance with the 3Rs principles in science and with law requirements in Brazil. In this process, it seems essential to engage different stakeholders in the discussions including laboratory staff, government and society. In future studies the DT could be applided to the reality of laboratory animal use in other countries. We believe this application would require some adaptation of the DT structure, specially regarding the Cost, Normative and Governmental Branch.

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6 FINAL CONSIDERATIONS

In Brazil, it is mandatory to replace laboratory animals when alternative methods exist. This is established by the Brazilian Animal Protection legislation and, as seen in other countries, is increasingly required by society, which demands a more humane treatment of animals. The aim of this study was to create viable pathways to assist the implementation of Validated Alternative Methods for rabies diagnosis in Brazil, contributing to the reduction of harmful animal use.

Although in Brazil the implementation and use of alternative methods seems more recent comparing to countries such as the United Kingdom, Chapter II shows that there are several opportunities to replace laboratory animals in the country. However, Brazilian laboratories performing rabies diagnosis mainly use live animals for the Mouse Inoculation Test (MIT) to analyze suspect samples. Barriers that might hinder the replacement of animals for rabies diagnosis also seem to exist in other areas, including research, teaching and industry. Thus, it is necessary to know and address them to be able to implement alternatives, this way fulfilling the law and, more importantly, reducing unnecessary animal suffering.

Chapter III was dedicated to study the scenario of rabies diagnosis, motivated by the fact that despite the availability of alternative methods here represented by the Virus Isolation in Cell Culture (VICC), tens of thousands of animals are used for MIT every year. This animal use is higher in Brazil compared to other countries. Barriers to the replacement of laboratory animals identified in this study include lack of laboratory structure and lack of financial resources. The perceived high cost of *in vitro* methods was one of the reasons most frequently pointed out by Brazilian respondents, while their low cost was one of the reasons mentioned by most of the non-Brazilian respondents for employing alternatives. Because details on the full costs of the different diagnostic methods may contribute to the implementation of alternative methods, the cost paradox found was then further studied.

By comparing the costs of MIT and VICC it was confirmed that using live animals costs more than the alternative method for rabies diagnosis in Brazil. So, the study proposed by Chapter IV can contribute for the resolution of barriers to laboratory animal replacement, especially those related to costs. Since the high cost of alternative methods is commonly used as a justification for not replacing animals in

several situations, the present study may be applied to other scenarios other than the rabies diagnosis to contribute to the implementation of such methods.

Following the example of the cost comparison study, other barriers that hinder the replacement of animals for rabies diagnosis were also addressed. Based on the previous studies, Chapter IV describes a decision tree framework that can be applied to scenarios of laboratory animal use where alternative methods exist. The proposed framework can contribute to increase compliance with the 3Rs principles in science, specially the one regarding Replacement, and with law requirements to such replacement in Brazil.

It is hoped that the results of the present thesis be applied to different scenarios of laboratory animal use and that in the near future it will be possible to see a decrease in numbers of animals used and an increase in the use of alternative methods in Brazil. The presented strategies may be considered useful tools to oppose the triad composed by poor philosophical debate regarding the treatment of animals, poor cost analysis regarding alternative methods as well as reducionist scientific thinking.

APPENDIX I

LIST OF MATERIAL PRODUCED FOR PUBLICATION RELATED TO THE THESIS CHAPTERS

CHAPTER	DISCLOSURE PLACE	KIND OF MATERIAL	SITUATION	APPENDIX
II	*Veterinária em Foco* journal	Scientific paper	Published in 2012	
III	Alternatives to Laboratory Animals journal	Scientific paper	Published in 2014	
	I Latin American Congress on Alternatives to Animal Use in Education, Research and Industry	Abstract	Published and presented as a poster, 2012	II
	Proceedings of the 39th *Congresso Brasileiro de Medicina Veterinária* (CONBRAVET)	Abstract	Published and presented as a talk by Carla Forte Maiolino Molento, 2012	III
	Proceedings of the Universities Federation for Animal Welfare (UFAW) International Animal Welfare Science Symposium	Abstract	Published and presented as a talk, 2013	IV
IV	Alternatives to Laboratory Animals journal	Scientific paper	Submitted in August 2014	
	Proceedings of the *Congresso Medvep de Especialidades Veterinárias*	Extended abstract	Published and presented as a poster by Carla Forte Maiolino Molento, 2013	V
	Proceedings of the 9th World Congress on Alternatives and	Abstract	Published and presented as a poster, 2014	VI

	Animal Use in the Life Sciences			
V		Scientific paper	To be submitted	
	Proceedings of the III *Congresso Brasileiro de Bioética e bem-estar Animal*	Extended abstract	Published and presented as a poster, 2014	VII
	Proceedings of the 9th World Congress on Alternatives and Animal Use in the Life Sciences	Abstract	Published and presented as a poster, 2014	VIII
III, IV, V	*Laboratório de Bem-estar Animal* (LABEA) Newsletter	Newsletter	Published, 2012	IX
	Proceedings of the Universities Federation for Animal Welfare (UFAW) International Animal Welfare Science Symposium, 2013	Abstract	Published and presented as a poster	X
	Laboratório de Análises Socioeconômicas e Ciência Animal (LAE) Newsletter	Newsletter	Published, 2013	XI

APPENDIX II

THE USE OF ALTERNATIVE METHODS FOR RABIES DIAGNOSIS IN BRAZIL

BONES, V. C.[1]; WEARY, D. M.[2]; MOLENTO, C. F. M.[1]

[1] Animal Welfare Laboratory (LABEA), Agrarian Sciences Sector (SCA), Federal University of Paraná (UFPR), Curitiba- PR, Brazil. [2] Animal Welfare Program, Faculty of Land and Food Systems, University of British Columbia (UBC), Vancouver, Canada.

The use of laboratory animals is common practice but is also a source of increasing public concern. Some types of animal use can be replaced using *in vitro* methods, such as the use of a cell culture (CC) instead of the mouse inoculation test (MIT) for rabies diagnosis. The objective of this work was to describe methods for rabies diagnosis in Brazil in comparison to other countries, using a web forum. Between December 2011 and March 2012, 486 people working with rabies diagnosis in different countries were invited by e-mail to participate in the forum, to describe the methods they used and their reasons for using them. Thirty-five English-speaking and 12 Portuguese-speaking respondents answered the questions; 11 Portuguese-speaking respondents worked in Brazil. Six Portuguese-speaking and seven English-speaking respondents used the MIT. The high cost for introducing the CC was mentioned as a limitation by one English-speaking respondent and by five Portuguese-speaking respondents. The Brazilian Federal Act 9605/1998 states that animal experimentation is a crime when alternative methods exist. The Brazilian Health Ministry guidelines recognize that, once properly implemented in the laboratory, the CC is more economic and efficient than the MIT. The results of this study suggest the proportion of laboratories using mice to perform rabies diagnosis in Brazil is high and may conflict with Brazilian law and the Health Ministry recommendations.

Keywords: Animal welfare, Laboratory animals, Replacement

APPENDIX III

AS BARREIRAS À SUBSTITUIÇÃO DO USO DE ANIMAIS PARA O DIAGNÓSTICO DA RAIVA NO BRASIL

BONES, V. C.[1]; WEARY, D. M.[2]; MOLENTO, C. F. M.[1]

[1] Laboratório de Bem-estar Animal (LABEA), Setor de Ciências Agrárias (SCA), Universidade Federal do Paraná (UFPR), Curitiba, PR, Brasil.
[2] Animal Welfare Program, Faculty of Land and Food Systems, University of British Columbia (UBC), Vancouver, Canadá.
Endereço eletrônico para correspondência: carlamolento@ufpr.br

Milhões de animais são utilizados em laboratórios; porém, há uma preocupação crescente da sociedade com o sofrimento animal. Para diagnóstico da raiva, por exemplo, no Brasil é comum a utilização do teste do isolamento viral em camundongo (IVC), no qual amostra de indivíduo suspeito é inoculada em cérebro de camundongos saudáveis, embora o IVC possa ser substituído por métodos *in vitro* internacionalmente validados desde a década de 80, como o isolamento viral em cultura de células (IVCC). O objetivo deste trabalho foi descrever as barreiras à utilização de métodos *in vitro* para o diagnóstico da raiva no Brasil utilizando uma plataforma online. De dezembro de 2011 a agosto de 2012, 129 brasileiros que trabalham com diagnóstico da raiva foram convidados a participar do estudo descrevendo as barreiras que impedem a utilização de alternativas neste cenário. Doze pessoas aceitaram o convite; suas respostas foram analisadas qualitativamente e classificadas em grupos de comentários semelhantes, os quais constituem as barreiras. Cada resposta poderia conter mais de um comentário, portanto o número de barreiras é maior que o número de participantes. As barreiras mencionadas e suas frequências absolutas foram: falta de recursos humanos e capacitação profissional (5); acomodação, hábito e falta de boa vontade das pessoas (4); falta de recursos financeiros (3); barreiras regulatórias e falta de incentivo do governo (3); barreiras cultural e ética (3); falta de estrutura dos laboratórios, equipamentos e materiais (2); falta de conhecimento e conscientização (2); importância dos fatores orgânicos para observação da doença (2); baixa sensibilidade ou falhas das técnicas *in vitro* (1); facilidade e baixo preço do IVC (1); falta de tempo (1). De forma geral, as barreiras percebidas pelos respondentes denotam falta de investimento e iniciativa institucionais, bem como resistência das pessoas envolvidas. Importante ressaltar que a Lei Federal 9605/1998 determina que é crime realizar experimentos em animais quando existirem métodos alternativos e o Ministério da Saúde reconhece que, uma vez implementado, o IVCC é mais econômico e eficiente que o IVC. Os resultados sugerem que há oportunidade para aumentar a adoção de alternativas, pois algumas barreiras percebidas são imaginárias e outras são reais, mas passíveis de solução.

APPENDIX IV

CONSTRAINTS FOR THE ADOPTION OF ALTERNATIVE METHODS FOR RABIES DIAGNOSIS

VC Bones[1]; D Weary[2] and CFM Molento[1]

[1] Animal Welfare Laboratory, Department of Animal Sciences, Federal University of Paraná, Brazil
[2] Animal Welfare Program, Faculty of Land and Food Systems, University of British Columbia, Canada.

The use of laboratory animals is common practice but is also a source of increasing public concern. Some types of animal use may be replaced using *in vitro* methods, such as Cell Culture (CC) instead of the Mouse Inoculation Test (MIT) for rabies diagnosis. Our objective was to describe the use of the MIT and alternative methods for rabies diagnosis in Brazil and other countries, and identify barriers to replacing *in vivo* diagnostic tests. Between 2011 and 2012, 484 people working with rabies diagnosis in different countries were invited to participate in an online forum called "Your Views on the Use of Animals for Rabies Diagnosis". Twelve Brazilians and 43 non-Brazilians replied. Non-Brazilians were from United States (6), Canada (5), India (4), South Africa (3), Italy (3), and other 22 countries. Nine (75%) Brazilian and 14 (32%) non-Brazilian respondents indicated that they used the MIT. People were asked to explain why they used their method of choice; their comments were then classified as either for or against the use of alternative methods. Thirty-nine percent of the comments from Brazilians and 80% from non-Brazilians expressed support to the use of alternative methods. Both Brazilian and non-Brazilian respondents described barriers to the implementation of alternative methods; the perceived barriers included high cost to implement CC, lack of structure, equipment or materials in the laboratories, lack of qualified staff, resistance to change, regulatory barriers and lack of incentive by the government. The lack of constraints to replace the use of animals was mentioned by six non-Brazilian and none of the Brazilian respondents; this might indicate that the Brazilian participants perceive more difficulties in pursuing this change. This perception may be related to resistance to change, conflicting with Brazilian law and the Brazilian Health Ministry recommendations. Results suggest that most Brazilian laboratories are using the MIT, and that understanding the barriers to adopting CC may facilitate change in Brazil and elsewhere around the world.

APPENDIX V

METODOLOGIA PARA COMPARAÇÃO DE CUSTOS ENTRE MÉTODOS *IN VIVO* E *IN VITRO* PARA O DIAGNÓSTICO DA RAIVA

Vanessa Carli Bones, Médica Veterinária, Mestre, Laboratório de Bem-estar Animal (LABEA), Universidade Federal do Paraná (UFPR)
Augusto Hauber Gameiro, Engenheiro Agrônomo, Doutor, Laboratório de Análises Socioeconômicas e Ciência Animal (LAE), Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo (FMVZ-USP) campus Pirassununga
Juliana Galera Castilho, Bióloga, Doutora, Instituto Pasteur de São Paulo
Carla Forte Maiolino Molento, Médica Veterinária, Doutora, LABEA/UFPR. E-mail para correspondência: carlamolento@ufpr.br

Resumo: Para o diagnóstico da raiva recomenda-se a confirmação dos resultados da Imunofluorescência Direta por meio do Isolamento Viral em Camundongos (IVC) ou do Isolamento Viral em Cultivo Celular (IVCC). Em virtude de impedimentos éticos e legais da utilização de animais quando existem alternativas, e da justificativa para o uso de animais ser com frequência baseada em aspectos de custo, o objetivo deste trabalho foi propor um método para estudar comparativamente os custos de realização do IVC e do IVCC para o diagnóstico da raiva. O estudo baseou-se em um acompanhamento da rotina laboratorial do Instituto Pasteur de São Paulo. Tal acompanhamento possibilitou a organização de listas de itens necessários para a realização de ambos os testes e a proposição de uma metodologia que permitirá um estudo detalhado dos custos do IVC e do IVCC para a realidade brasileira. A aplicação de tal metodologia poderá contribuir para a resolução de um obstáculo à substituição do uso de animais para o diagnóstico da raiva no Brasil.
Palavras-chave: Animais de laboratório, bem-estar animal; substituição de animais; valor econômico.

Introdução:

A raiva leva a óbito em média 55.000 pessoas por ano e está reemergindo como um sério problema de saúde pública na África, Ásia e América Latina (1). O seu diagnóstico laboratorial é fundamental para a confirmação de casos em seres humanos e animais. Para tal se recomenda o teste de Imunofluorescência Direta (IFD) e a confirmação com Isolamento Viral em Camundongos (IVC) ou Isolamento Viral em Cultivo Celular (IVCC) (1). Alternativas ao uso de animais como o IVCC apresentam bons resultados e são mais adequadas em termos de bem-estar animal por evitar sofrimento desnecessário (1). Ainda, tais métodos apresentam menor custo em relação ao uso de animais (1). De forma geral, para o diagnóstico em questão, o IVCC custa aproximadamente cinco vezes menos que o IVC (2). Apesar das informações constantes na literatura internacional, nossos dados preliminares sugerem uma percepção generalizada de que o uso de animais apresente menor custo no Brasil. O objetivo deste trabalho foi propor um método para estudar comparativamente os custos de realização do IVC e do IVCC para o diagnóstico da raiva no Brasil.

Material & Métodos:

O presente estudo parte do princípio da construção de um laboratório modelo de diagnóstico da raiva e se baseia em um acompanhamento da rotina de

diagnóstico da raiva realizada no Instituto Pasteur de São Paulo, em novembro de 2012. Tal acompanhamento permitiu a organização de itens variáveis e fixos para IVC e para IVCC. Os custos podem ser variáveis (*CV*) e fixos (*CF*) em função de sua relação com a quantidade produzida (3). O *CV* é composto por itens que variam em função da quantidade produzida; assim, quanto maior o número de exames diagnósticos realizados, maior será o consumo de reagentes, por exemplo. O *CF* por sua vez não se altera, independente da quantidade de exames realizados em um determinado período, como por exemplo, o aluguel, a depreciação de equipamentos e os salários.

Resultados e discussão:

Foram sistematizadas quatro categorias de itens necessários para a realização dos testes, sendo que foram listados 25 itens variáveis e 47 fixos para o IVC, totalizando 72 itens, e 38 itens variáveis e 63 fixos para o IVCC, 101 no total. O número de itens para IVC é menor do que para IVCC; porém, tal resultado não significa, necessariamente, que o IVC apresente menor custo de realização. A diferença pode estar relacionada ao fato de que para o IVC foi considerada a compra de camundongos e não a sua criação no biotério do laboratório de diagnóstico. Itens variáveis foram classificados em subcategorias como: reagentes, materiais inerentes aos testes e ao local, e custos associados a materiais de segurança de técnicos de laboratório; os fixos incluíram depreciação de equipamentos, materiais de apoio inerentes aos testes e ao local, custos associados à mão de obra e metragem das instalações. Não foram listados itens considerados de apoio, pois são de uso comum a ambos os testes, a exemplo do sistema de recolhimento de lixo e dos geradores de energia. Somente foram incluídos itens de apoio cuja intensidade de uso seja diferente de maneira marcante entre os testes, como no caso da autoclave que processa um volume maior de material por semana oriundo do IVC do que do IVCC. Para facilitar a realização do estudo os custos foram denominados Custo Variável do IVC (*CVIVC*), Custo Fixo do IVC (*CFIVC*), Custo Variável do IVCC (*CVIVCC*) e Custo Fixo do IVCC (*CFIVCC*); tais custos compõem a fórmula que posteriormente possibilitará o cálculo do Custo Total Médio por Amostra (*CTMe*) de ambos os testes e comparação entre eles:

$$CT = CVMe.n + CF$$
$$CTMe = CT / n$$

Onde *CT* é Custo Total; *CVMe* é o Custo Variável Médio por Amostra, que se refere à *CVIVC* e *CVIVCC* divididos pelo número de amostras processas em cada teste; *n* é o número de amostras processadas em cada teste; *CF* é o Custo Fixo de ambos os testes, ou seja, *CFIVC* e *CFIVCC*.

Além do *CTMe*, será possível analisar os custos dos testes IVC e IVCC em duas perspectivas: 1) em relação à implantação dos métodos, supondo que o laboratório ainda não realize o diagnóstico da raiva rotineiramente mas deseja implantá-lo; para tal é considerado o *CT*; 2) a utilização rotineira dos métodos de diagnóstico, supondo que no laboratório os materiais e equipamentos permanentes estejam disponíveis; para tal é considerado apenas o *CV*. Desta forma, a metodologia proposta permitirá a comparação dos custos do IVC e do IVCC em cenários de implantação e de rotina laboratorial.

Conclusões:

O método proposto neste trabalho poderá contribuir para a análise dos custos do IVC e do IVCC para o diagnóstico da raiva, permitindo a comparação específica em cenários de implantação e naqueles em que a comparação é apenas entre as rotinas laboratoriais de cada teste. Somente por meio da implantação de um método detalhado de comparação de custos a controvérsia entre a literatura internacional e a percepção nacional sobre os custos do IVC e do IVCC poderá ser resolvida.

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APPENDIX VI

COST COMPARISON BETWEEN THE MOUSE INOCULATION TEST (MIT) AND THE VIRUS ISOLATION IN CELL CULTURE (VICC) FOR RABIES DIAGNOSIS

Bones V.C.[1], Gameiro A.H.[2], Castilho J.G.[3], Molento C.F.M.[1]

[1]Animal Welfare Laboratory (LABEA), Animal Sciences Department, Federal University of Paraná (UFPR), Curitiba, Brazil; [2]Socioeconomic Analysis and Animal Science Laboratory (LAE), Faculty of Veterinary Medicine and Animal Sciences (FMVZ), University of São Paulo (USP) Pirassununga campus, Pirassununga, Brazil; [3]Pasteur Institute of São Paulo, São Paulo, Brazil.

Because the decision for using laboratory animals is frequently based on cost aspects (1), our objective was to compare the costs to perform the Mouse Inoculation Test (MIT) and the Virus Isolation in Cell Culture (VICC) for rabies diagnosis in Brazil. Based on the observation of laboratory routine at Pasteur Institute, São Paulo, we listed fixed and variable cost items (2) necessary to perform both tests. We calculated the average total cost per sample and the costs of 1) implementation, and 2) routine use of both diagnostic tests. Considering that 200 MIT tests are equivalent to 350 VICC tests in terms of facilities and staff hours needed per month, one sample analyzed by MIT costs around 193% more than by VICC. MIT is also 67% and 406% more expensive than VICC considering implementation and variable costs for routine use per month, respectively. Such variations are mainly due to the higher cost of MIT variable items, as the animals themselves (76% of variable cost). Our results contribute to the resolution of cost obstacles that hinder the replacement of laboratory animals for rabies diagnosis in Brazil. The presented methodology may be useful for other situations of animal use when validated alternatives exist.

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APPENDIX VII

ÁRVORE DE DECISÃO PARA FACILITAR A SUBSTITUIÇÃO DE ANIMAIS DE LABORATÓRIO NO BRASIL

Vanessa Carli Bones[3] & Carla Forte Maiolino Molento[1]

INTRODUÇÃO

Um conceito reconhecido mundialmente por nortear a utilização de animais de laboratório é o chamado Princípio dos 3Rs, do inglês Substituição, Redução de animais e Refinamento das técnicas envolvendo animais (RUSSELL & BURCH, 1992). A necessidade de substituição de animais de laboratório pode ser justificada pelo sofrimento animal envolvido na manutenção e nos procedimentos experimentais, pelo fato de que utilização de animais parece custar mais que o uso de métodos alternativos validados (MAV) e pelo fato de que a Legislação de Proteção Animal brasileira (BRASIL, 1998) proíbe o uso de animais de laboratório quando existirem recursos alternativos. Tal lei está de acordo com a Diretiva Europeia 2010/63/EU (EUROPEAN COMMISSION, 2010), a qual estabelece que o uso de animais para propósitos científicos e didáticos somente deve ser considerada quando alternativas que não provenham de fontes animais estejam indisponíveis. O objetivo do presente trabalho foi descrever o desenvolvimento de uma estrutura em árvore de decisão (AD) para auxiliar a substituição de animais de laboratório no Brasil.

METODOLOGIA

Para facilitar a tomada de decisões em cenários de potencial substituição de animais de laboratório foram propostas estratégias, estruturadas em uma AD, com auxílio do programa Dia Portable®. A AD proposta apresenta sugestões de como superar obstáculos que podem impedir a substituição de animais de laboratório, baseadas nas barreiras mais citadas em trabalho publicado utilizando como modelo o diagnóstico da raiva (BONES et al, 2014). Segundo SHAH HAMZEI & MULVANEY (1999), a AD é uma estrutura geralmente organizada de cima para baixo e consiste em um número finito de nós contendo informações conectadas por meio de linhas. Os nós que compõem a estrutura incluem questões, sendo uma delas a principal, pontos de decisão, recomendações e pontos finais. Os nós foram conectados utilizando linhas e, a partir da questão principal, grupos de nós formaram ramos ou sub-ramos. Para facilitar a visualização da estrutura, as diferentes categorias de nós foram representados por caixas de diferentes formatos e os sub-ramos que derivam do ramo principal foram coloridos em escala de cinza.

RESULTADOS E DISCUSSÃO

A primeira questão da AD se refere à existência de MAV para um dado cenário de utilização de animais de laboratório. Se não (N) existirem MAV, é necessário planejar o seu desenvolvimento. Tal planejamento envolve, entre outras ações, a obtenção de recursos financeiros, por exemplo, por meio de submissão de projetos a agências oficiais de financiamento de pesquisa; a recomendação de obtenção de recursos financeiros aparece em outros pontos da AD. Enquanto o

[3] Laboratório de Bem-estar Animal (LABEA), Universidade Federal do Paraná (UFPR), Rua dos Funcionários, 1540, Curitiba, Brasil. Autora para correspondência: vcb.vete@gmail.com

MAV está sendo desenvolvido é necessário submeter projetos envolvendo o uso de animais para Comitês de Ética no Uso de Animais institucionais (BRASIL, 2008). Para aumentar a consideração e o uso do princípio dos 3Rs (RUSSEL & BURCH, 1992), as comissões de ética no uso de animais devem exigir aos proponentes que comprovem a inexistência de métodos alternativos antes de planejar a utilização de animais para pesquisa. Se MAV existem, a pessoa responsável pelo laboratório (PR) conhece tal método? Se N, ela deve conhecê-lo por meio de pesquisa em sítios eletrônicos especializados ou contratação de um serviço de consultoria em métodos alternativos. Se S, a PR está motivada a mudar? Se N, é necessário saber se a mesma conhece as leis brasileiras de proteção animal (BRASIL, 1998). Se N, é necessário expor a lei à PR. Se após tal exposição a PR não está motivada a mudar, é necessário denunciar o laboratório por estar infringindo a lei e em seguida encaminhá-lo ao Ramo Principal, pois a mudança é obrigatória. Se a PR está motivada a mudar, então ela deve ser encaminhada ao Ramo Principal. Se a PR conhece as leis brasileiras de proteção animal (BRASIL, 1998) e mesmo assim não está motivada a mudar, é necessário denunciar o laboratório a instâncias apropriadas e encaminhar o leitor ao Ramo Principal, uma vez que a mudança em cumprimento à lei é obrigatória. O encaminhamento do leitor ao Ramo Principal faz com que as possíveis barreiras sejam avaliadas e eliminadas até se alcançar a implantação do MAV.

Se a PR está motivada a mudar, ela passa a avaliar o Ramo Principal, o qual contém as barreiras mais frequentemente citadas por respondentes brasileiros e não-brasileiros, de acordo com BONES et al (2014). Então, no Sub-ramo dos Custos, os custos do MAV foram calculados? Se N, é necessário aplicar um método de comparação de custos. Se S, e porventura os custos do VAM são maiores que o uso de animais tanto para implantação de toda a estrutura do laboratório quanto para o uso rotineiro, é necessário obter recursos financeiros para adquirir a estrutura e os materiais. Se os custos do MAV são menores que o uso de animais, a implantação deve ser iniciada. No Sub-ramo dos Recursos Humanos, existem recursos humanos e qualificação profissional? Se N, é necessário obter recursos financeiros para contratar e treinar profissionais; se S, a implantação do MAV deve ser iniciada. No Sub-ramo denominado Resistência, há resistência por parte da equipe que utiliza animais no laboratório? Se N, a implantação do MAV deve ser iniciada. Se S, é necessário obter recursos financeiros para, por exemplo, desenvolver cursos que venham a discutir temas como 3Rs e ética no uso de animais, bem como expor a lei de proteção animal (BRASIL, 1998). E no Sub-ramo Normativo, há incompatibilidade entre leis de proteção animal e normas específicas que regem o uso de animais em estudo? Se N, a implantação do MAV deve ser iniciada. Se S, é necessário denunciar as normas específicas que permitem a utilização de animais a instâncias apropriadas.

CONCLUSÃO

A AD contém sugestões que podem auxiliar pessoas a superar os principais obstáculos que impedem a substituição de animais de laboratório por métodos alternativos em cenários onde tais recursos existem. Passo a passo, todos os ramos da árvore levam as pessoas a superar tais obstáculos à substituição dos animais e, necessariamente, à implantação de MAV ou ao seu desenvolvimento, no caso de ainda não existirem. Além de possibilitar sua aplicação em todos os cenários de utilização de animais de laboratório para os quais existem MAV, a AD também prevê a aplicação por qualquer pessoa interessada em implantar tais métodos.

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APPENDIX VIII

A DECISION TREE TO FACILITATE THE REPLACEMENT OF LABORATORY ANIMALS IN BRAZIL

Bones, V.C., Molento, C.F.M.

Animal Welfare Laboratory (LABEA), Animal Sciences Department, Federal University of Paraná (UFPR), Curitiba, Brazil.

We aimed to develop a decision tree to facilitate validated alternative methods (VAM) implementation. First, does a VAM exist? If yes (Y), is the laboratory director (LD) motivated to change? If Y, is/are there: Branch 1 (B1) Knowledge about VAM costs?; (B2) Qualified human resources?; (B3) Resistance to change by the staff?; and (B4) Incompatibilities between specific norms and Brazilian Animal Protection Law (1)?. For B1, if Y and VAM costs less than animal use, go to B2; if no (N), costs should be studied. For B2, if Y, go to B3; if N, training should be sought. For B3, if Y, staff should be educated about law, ethics and the 3Rs (2); if N go, to B4. For B4, if Y, norms should be denounced to appropriate instances; if N, implement VAM. If LD is unmotivated, does he/she know Brazilian Animal Protection Law (1)? If Y, law enforcement is required; if N, LD should be educated about law, ethics and the 3Rs (2). If LD becomes motivated, he/she is ready to move to B1. If a VAM is not available, it should be developed. This decision tree provides guidance to address the main obstacles for laboratory animal replacement (3).

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APPENDIX IX

Laboratório de Bem-estar Animal da Universidade Federal do Paraná

LABEA/UFPR

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O trabalho do LABEA/UFPR no contexto das alternativas ao uso de animais de laboratório no Brasil

Vanessa Carli Bones, Médica Veterinária, MSc em Ciências Veterinárias, Doutoranda pelo LABEA/UFPR, Curitiba-PR. E-mail: vcb.vete@gmail.com
Carla Forte Maiolino Molento, Médica Veterinária, MSc, PhD, Professora de Bem-estar Animal e Coordenadora do LABEA/UFPR, Curitiba-PR.
E-mail: carlamolento@ufpr.br

No Brasil, a exemplo de outros países, verifica-se uma crescente preocupação de cientistas, da indústria e da sociedade acerca da utilização de animais de laboratório. Nesse sentido, recentemente o governo brasileiro iniciou o processo de criação da Rede Nacional de Métodos Alternativos (Renama), cuja estrutura está focada na coordenação de desenvolvimento, certificação e validação de alternativas ao uso de animais. A Renama também coordenará o Centro Brasileiro de Validação de Métodos Alternativos (BraCVAM) e ambos os órgãos integrarão o Conselho Nacional de Controle de Experimentação Animal (CONCEA), criado pela Lei Federal n° 11.794 e coordenado pelo Ministério da Ciência, Tecnologia e Inovação (MCTI).

Neste contexto, o LABEA/UFPR, mantém uma linha de pesquisa dedicada ao bem-estar de animais de laboratório, cujos trabalhos iniciaram em 2007. Um dos trabalhos integrantes de tal linha de pesquisa refere-se ao auxílio à implantação de alternativas no Brasil, usando como modelo de estudo a substituição de animais para o diagnóstico da raiva, intitulado Subsídios à Implantação de Métodos Alternativos Validados para o Diagnóstico da Raiva, em execução no âmbito de projeto de doutoramento da primeira autora.

O referido trabalho compreende a realização de um estudo utilizando uma plataforma online para descrever os métodos utilizados para o diagnóstico da raiva no Brasil e em outros países, e as barreiras à utilização de alternativas em tal cenário; um comparativo de custos entre as provas de Isolamento Viral em Camundongos e de Inoculação Viral em Cultivo Celular para o diagnóstico da raiva; a implantação de método alternativo para o diagnóstico; bem como a sensibilização de pessoas que trabalham com animais de laboratório e a ampliação da disponibilidade de informações acerca de questões relativas aos métodos alternativos.

O LABEA tem participado de eventos importantes nacionais e internacionais relacionados ao tema (Figura 1). Em 2009, durante o 7º Congresso Mundial de Métodos Alternativos nas Ciências da Vida (*7th World Congress on Alternatives and Animal Use in the Life Sciences*), em Roma, foi apresentado o resumo *Animal use in research in Brazil*. Em 2011, durante o *8th World Congress on Alternatives and*

Animal Use in the Life Sciences, em Montréal, foi apresentado o trabalho *Perception of animals used in education and research in Brazil by students and professors*. Em 2012, durante o I Congresso Latino-Americano de Métodos Alternativos ao Uso de animais no Ensino, Pesquisa e Indústria (COLAMA), em Niterói, foi apresentado o trabalho *The use of alternative methods for rabies diagnosis in Brazil*, mostrando que a proporção de laboratórios que utilizam camundongos para o diagnóstico da raiva no país é grande em comparação a outros países e este fato conflita com a legislação federal. Também importante foi o 39º Congresso Brasileiro de Medicina Veterinária (CONBRAVET), em Santos, ocasião em que foi apresentado o trabalho As barreiras à substituição do uso de animais para o diagnóstico da raiva no Brasil, demonstrando que as barreiras denotam falta de investimento e iniciativa institucionais, bem como resistência das pessoas envolvidas, sugerindo que há oportunidade para aumentar a adoção de alternativas; tal trabalho recebeu o prêmio de Melhor Trabalho Científico Apresentado.

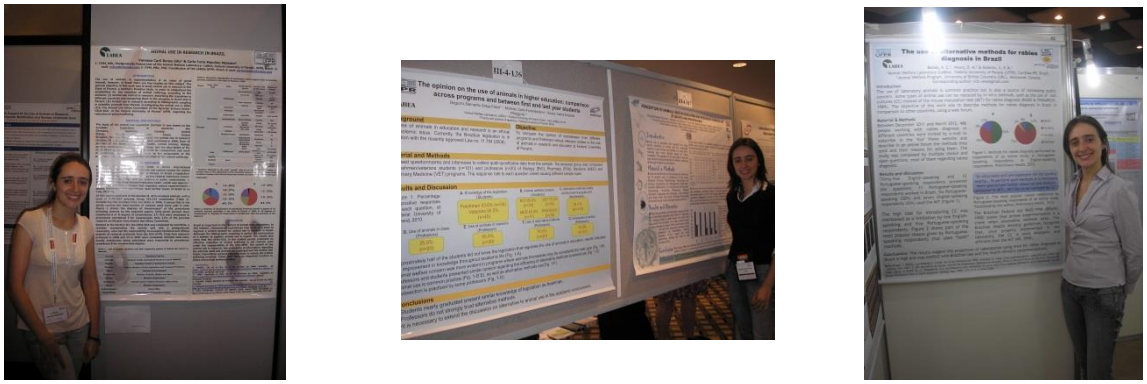

Figura 1. Fotos representando a participação da aluna Vanessa Carli Bones em conferências internacionais relacionadas à utilização de métodos alternativos. 7º Congresso Mundial de Métodos Alternativos, Roma, 2009; 8° Congresso Mundial de Métodos Alternativos, Montreal, 2011; 1º Congresso Latino-Americano de Métodos Alternativos ao Uso de animais no Ensino, Pesquisa e Indústria, Niterói, 2012; respectivamente.

APPENDIX X

IMPLEMENTATION OF VALIDATED ALTERNATIVES TO LABORATORY ANIMAL USE IN BRAZIL: RABIES DIAGNOSIS AS A MODEL

VC Bones[1]; JG Castilho[2]; AH Gameiro[3]; RX Anthony[4]; CFM Molento[1]

[1] Animal Welfare Laboratory, Department of Animal Sciences, Federal University of Paraná, Brazil
[2] Pasteur Institute of São Paulo, Brazil
[3] Laboratory of Socioeconomic Analysis and Animal Science, Faculty of Veterinary Medicine and Animal Science, University of São Paulo, Pirassununga, Brazil
[4] Department of Philosophy, University of Alaska Anchorage, Alaska

The use of laboratory animals is common for practices such as teaching, research and disease diagnosis, even though there are alternative methods internationally validated to replace some of them. The Animal Welfare Laboratory at UFPR is developing a study to foster the implementation of validated alternative methods, using the replacement of animal use for rabies diagnosis as a model. This study aims to contribute to the technological autonomy, staff development and qualification in the area of alternatives to animal use, as well as to contribute to the construction of the Brazilian National Network for Alternative Methods, a recent initiative of the Brazilian government. The first part of this study is the development of an economic cost comparison between two methods for rabies diagnosis, the *in vivo* Mouse Inoculation Test (MIT) and the *in vitro* Cell Culture (CC). The second part is the implementation of the alternative method for rabies diagnosis in a Brazilian laboratory. Finally, the third part is a consciousness-raising process, involving the engagement of laboratory staff with animal ethics and the need for adoption of alternative methods, and the dissemination of information regarding suitable alternative methods. This work is expected to: (a) increase knowledge regarding the economic cost of methods when comparing the MIT and the CC for rabies diagnosis; (b) implement the alternative method for rabies diagnosis in a Brazilian laboratory that currently uses MIT; (c) lead to a list of factors that hinder the use of alternatives and propose resolution of such factors; (d) generalize knowledge obtained to other testing scenarios; (e) support ethical discussions around the use of laboratory animals and alternative methods, and (f) stimulate the use of alternatives to laboratory animals in general.

APPENDIX XI

AS POSSIBILIDADES E OS DESAFIOS RELACIONADOS À ADOÇÃO DE ALTERNATIVAS AO USO DE ANIMAIS DE LABORATÓRIO NO BRASIL

Vanessa Carli Bones, Médica Veterinária, Mestre, doutoranda, Laboratório de Bem-estar Animal (LABEA), Universidade Federal do Paraná (UFPR)
Carla Forte Maiolino Molento, Médica Veterinária, Doutora, LABEA/UFPR. E-mail para correspondência: carlamolento@ufpr.br

A utilização de animais de laboratório em cenários como ensino, pesquisa, testes de cosméticos, testes e produção de medicamentos e diagnóstico de doenças que acometem seres humanos e animais é uma prática comum em diversos países. Neste sentido, Taylor e colaboradores (2008) estimaram a utilização de 115.3 milhões de animais em experimentação em 179 países e de 1,16 milhão de animais vertebrados no Brasil em 2005, correspondendo a 11ª posição entre os países que mais utilizam animais de laboratório no mundo. De forma semelhante, Silla e colaboradores (2010) investigaram o uso de animais em pesquisa no Brasil por meio do método de amostragem bibliográfica, a partir de 45% dos periódicos científicos que envolvem pesquisa animal publicados no estado do Paraná em 2006; os resultados mostram um total, estimado por um cálculo conservador, de 3.497.653 animais usados, dos quais 216.223 foram vertebrados. Tais publicações sugerem que o Brasil seja importante no contexto mundial do uso de animais de laboratório.

Muitas formas de utilização de animais de laboratório podem ser substituídas por métodos alternativos, ou em situações em que a substituição não é completa, o número de animais pode ser reduzido a um mínimo possível, assim como podem ser utilizadas formas de refinamento dos procedimentos com o intuito de reduzir a dor e o sofrimento envolvido. Estas premissas constituem o chamado conceito dos 3Rs, do inglês *Replacement*, *Reduction* e *Refinement* (RUSSEL; BURCH, 1992), que compreende a Substituição de animais, a Redução do número de animais e o Refinamento dos procedimentos envolvendo animais.

Neste sentido, o Laboratório de Bem-estar Animal (LABEA) da Universidade Federal do Paraná (UFPR) está desenvolvendo um estudo cujo objetivo principal é auxiliar a implantação de métodos alternativos no Brasil, usando como modelo a substituição de animais vivos para o diagnóstico da raiva. O projeto, intitulado Subsídios à Implantação de Métodos Alternativos Validados para o Diagnóstico da Raiva, pretende entender os principais obstáculos à substituição do uso de animais nos cenários em que existem alternativas validadas, mas mesmo assim animais continuam sendo utilizados. O diagnóstico da raiva foi escolhido como modelo de estudo pois existe um método alternativo validado internacionalmente, o Isolamento Viral em Cultivo Celular (IVCC), que pode substituir o uso de animais vivos utilizados para a realização da Inoculação Viral em Camundongos (IVC). O IVCC é tão sensível quanto a IVC (WHO, 2005; MS, 2008; MAPA, 2009), é mais econômico (MS, 2008; MAPA, 2009; OIE, 2011) e gera resultados mais rápidos (WHO, 2005; MS, 2008; MAPA, 2009; OIE, 2011). A rapidez do diagnóstico da raiva por meio do IVCC é um importante fator de estímulo à implantação de tal método, uma vez que a doença não causa sinais clínicos facilmente identificáveis, desta forma tornando o diagnóstico laboratorial essencial. Ainda, a substituição do uso de animais é urgente, pois a IVC tem potencial de causar severo sofrimento animal.

No Brasil, a implantação de métodos alternativos ao uso de animais de laboratório é exigência legal. A Lei Federal brasileira n° 9.605 de 1998, ou Lei de Crimes Ambientais, trata do uso de animais em experimentação e determina penalização a quem realiza experiência dolorosa ou cruel em animal vivo ainda que para fins didáticos ou científicos, quando existirem recursos alternativos (BRASIL, 1998). Apesar de tal exigência, parte dos resultados do estudo Subsídios à Implantação de Métodos Alternativos Validados para o Diagnóstico da Raiva demonstram que a proporção de pessoas que trabalham com o diagnóstico da raiva e que utilizam métodos *in vitro* no Brasil é proporcionalmente menor que em outros países (BONES, et al., 2012a, BONES et al., 2013a), conforme mostra a Figura 1.

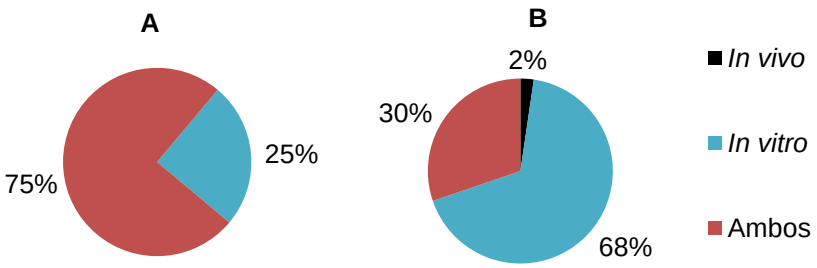

Figura 1. Métodos para o diagnóstico da raiva utilizados por 12 respondentes brasileiros (A) e 43 não brasileiros (B); estudo online sobre métodos alternativos para o diagnóstico da raiva, agosto de 2011 a agosto de 2012 (adaptado de BONES et al., 2013a).

Tal trabalho também demonstra que existe um paradoxo relacionado aos custos de implantação de métodos alternativos para o diagnóstico da raiva, pois parece haver uma tendência de que brasileiros percebam os métodos *in vitro* como sendo mais caros que os métodos *in vivo*, enquanto que não brasileiros tendem a aceitar mais a utilização de métodos alternativos por acreditar que estes sejam menos caros (BONES, et al., 2013a). Tal resultado motivou o início de uma parceria entre o LABEA e o Laboratório de Análises Socioeconômicas e Ciência Animal (LAE) da Universidade de São Paulo (USP), Campus Pirassununga, em 2012, com o objetivo de estudar comparativamente os custos do IVCC e da IVC especificamente no contexto brasileiro.

Dentre os resultados preliminares da parceria entre o LAE/USP e o LABEA/UFPR está a publicação de uma proposta de metodologia para comparação de custos entre os métodos *in vivo* e *in vitro* para o diagnóstico da raiva (BONES et al. 2013b), a qual se baseou em um acompanhamento da rotina laboratorial do Instituto Pasteur de São Paulo em novembro de 2012. Tal acompanhamento possibilitou a organização de listas de itens necessários para a realização de ambos os testes e a proposição da referida metodologia que permitirá a análise dos custos dos testes IVCC e IVC em duas perspectivas: em relação à implantação dos métodos, supondo que o laboratório ainda não realize o diagnóstico da raiva e deseje implantá-lo, bem como em relação à utilização rotineira de ambos os métodos de diagnóstico, supondo que no laboratório os materiais e equipamentos permanentes estejam disponíveis. Desta forma, a metodologia proposta permitirá a comparação dos custos do IVCC e da IVC em cenários de implantação e de rotina laboratorial. Um detalhado estudo comparativo de custos entre os métodos IVCC e IVC para o diagnóstico da raiva está em andamento com base na referida

metodologia, com o objetivo de contribuir para o esclarecimento da controvérsia entre as recomendações nacionais e internacionais e a percepção dos brasileiros sobre os custos do IVCC e da IVC.

De acordo com a opinião de pessoas que trabalham com o diagnóstico da raiva, além dos custos, vários fatores dificultam ou impedem a substituição de animais vivos por métodos alternativos, dentre eles a falta de estrutura, equipamentos e materiais nos laboratórios, bem como a falta de recursos humanos e qualificação profissional (BONES et al., 2013a). De forma geral, tanto no Brasil quanto em outros países as barreiras percebidas pelos respondentes denotam falta de investimento e iniciativa institucionais, bem como resistência das pessoas envolvidas (BONES, et al. 2012b; BONES et al., 2013a). Por exemplo, quando questionado sobre as barreiras que impedem a substituição de animais por métodos alternativos para o diagnóstico da raiva, um respondente não brasileiro que trabalha com diagnóstico da raiva respondeu:

… O cultivo celular pode ser caro, requer capelas de fluxo laminar e níveis consideravelmente altos de treinamento... ...Muitos laboratórios, especialmente na África, não possuem as instalações necessárias para cultivo celular ou os reagentes e as habilidades para realizar o teste da imunofluorescência direta, portanto a inoculação em camundongos é ainda amplamente utilizada. (adaptado de BONES et al. (2013a), resposta traduzida pelos autores)

A indicação de barreiras que impedem a substituição de animais vivos para o diagnóstico da raiva por parte das pessoas que trabalham diretamente em tal cenário demonstra que existem desafios a serem vencidos em tal processo. Um desafio importante do processo de implantação do método alternativo para o diagnóstico da raiva é vencer a resistência das pessoas a mudanças. De acordo com BORTOLOTTI e colaboradores (2008), como a mudança pressupõe algo novo, as pessoas têm dificuldade de quebrar paradigmas e mudar comportamentos, desta forma podendo se tornar inseguras e resistentes. Além disso, muitos cientistas podem apoiar o uso de animais por pensar que tudo aquilo que aprenderam é verdade absoluta e não tem tempo ou vontade para questionar se as suas atitudes são sujeitas a mudanças (GREEK & GREEK, 2004).

No âmbito do diagnóstico da raiva, talvez a resistência das pessoas possa ser diminuída por meio da demonstração das vantagens do IVCC em comparação à IVC, da concordância por parte da equipe do laboratório com tais vantagens e também da oferta de apoio técnico e logístico ao laboratório interessado em implantar o método alternativo. Desta maneira, a resistência pode ser gradativamente diminuída entre as pessoas envolvidas com o diagnóstico da raiva na rotina laboratorial, a diretoria das instituições e o governo que, no Brasil, define os testes que preferencialmente devem ser realizados nos laboratórios por meio da publicação de manuais técnicos e normas.

Além da resistência por parte das pessoas e instituições que trabalham com o diagnóstico da raiva, é possível observar resistência por parte de outros agentes envolvidos indiretamente com o serviço oferecido pelo laboratório. Considerando o alto número de animais de laboratório utilizados no Brasil e em outros países (TAYLOR et al., 2008; SILLA et al., 2010) as indústrias que produzem e comercializam materiais de laboratório se beneficiam do uso de animais (GREEK & GREEK, 2004), por exemplo, a partir da venda de itens utilizados para o alojamento e para os procedimentos científicos em si.

Outro desafio identificado no processo de implantação do método alternativo é a possível dessensibilização dos profissionais para com os animais. Da mesma forma como o presente estudo visa a substituição do uso de animais para o diagnóstico da raiva, observa-se uma tentativa de diminuição do número de animais em outros cenários. No ensino, por exemplo, a utilização de animais em aulas práticas do curso de medicina veterinária está gradativamente sendo substituída por métodos alternativos em várias instituições brasileiras. Neste sentido, Deguchi e colaboradores (2012) mencionam a necessidade de expandir a discussão sobre alternativas ao uso de animais no ambiente acadêmico, de forma a educar médicos veterinários e mantê-los sensíveis para com os animais durante o processo de formação. O que se espera é que os cursos de veterinária possam mudar seus modelos metodológicos de ensino em direção à formação de profissionais que reconheçam os animais como seres sencientes (ZANETTI et al., 2011).

Além dos alunos de medicina veterinária, todos os profissionais que porventura venham a trabalhar com animais durante a carreira devem apresentar sensibilidade ao fato de que os animais vertebrados utilizados em procedimentos diversos são capazes de sentir. A construção de um nível de educação humanitária que proporcione o reconhecimento dos animais vertebrados como seres sencientes pode ser favorecida por meio da oferta da disciplina de bem-estar animal para aqueles cursos envolvidos com manipulação de animais durante e após a formação profissional. No cenário específico do diagnóstico da raiva, parece necessária a promoção de reflexão ética sobre a utilização de animais de laboratório por meio da sensibilização das pessoas envolvidas com a prova biológica e a apresentação de detalhamento da IVCC como alternativa ao uso de animais.

É importante ressaltar que a substituição de animais de laboratório por métodos alternativos pode ser um processo lento. Esperamos que o estudo *Subsídios à Implantação de Métodos Alternativos Validados para o Diagnóstico da Raiva* colabore para uma diminuição gradativa do sofrimento animal, principalmente porque pretendemos generalizar os resultados obtidos, aplicando as principais conclusões a outras situações para as quais já existam métodos alternativos validados ao uso de animais vivos. Desta forma, esperamos que os desafios inerentes ao processo de substituição de animais de laboratório identificados no âmbito de tal estudo sejam mais bem compreendidos e, assim, mais facilmente superados.

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APPENDIX XII

Depreciation and maintenance of durable and semi-durable goods necessary to perform the Virus Isolation in Cell Culture (VICC), in Portuguese

Fixos	Valor unitário do bem (R$)	n necessário	Valor total dos bens (preço inicial) (R$)	Valor residual (0% ou 10%) (R$)	Vida útil	Depreciação de bens duráveis e semiduráveis (R$)	Taxa de manutenção de bens duráveis e semiduráveis (0%, 10% ou 20%) (R$)
Equipamentos e materiais para os testes							
agitador de tubos	315.00	1	315.00	-	120	2.63	0.26
agitador magnético	1,000.00	1	1,000.00	-	120	8.33	0.83
balança analítica	4,002.00	1	4,002.00	400.20	120	30.02	6.67
balança de precisão	1,826.35	1	1,826.35	-	120	15.22	3.04
bomba de vácuo	1,358.00	1	1,358.00	-	120	11.32	1.13
botijão de N líquido	4,918.00	1	4,918.00	491.80	240	18.44	-
cabine de segurança biológica	56,890.00	1	56,890.00	5,689.00	240	213.34	47.41
câmara de Neubauer	107.00	1	107.00	-	60	1.78	-
canaleta	1.20	10	12.00	-	60	0.20	-
centrífuga refrigerada sem caçapas de segurança	4,350.00	1	4,350.00	435.00	120	32.63	3.63
estufa de CO2	2,149.70	1	2,149.70	214.97	120	16.12	1.79
íma de agitador magnético	17.73	1	17.73	-	120	0.15	-
lâmpada para cabine de segurança biológica	532.72	1	532.72	-	11	48.43	-
lâmpada para microscópio de imunofluorescência	590.00	1	590.00	-	13	45.38	-
lâmpada para microscópio óptico comum	12.00	1	12.00	-	7	1.71	-
lâmpada para sistema de purificação de água Milli-Q Direct-Q3 UV®	776.60	1	776.60	-	12	64.72	-
mangueira de borracha para bomba de vácuo	0.96	1	0.96	-	60	0.02	-
manômetro para cilindro de CO2	130.68	1	130.68	-	120	1.09	0.11
micropipetador automático 0 a 10 ul	690.00	1	690.00	-	120	5.75	0.58

micropipetador automático 100 a 1000 ul	690.00	1	690.00	-	120	5.75	0.58
micropipetador automático 20 a 200 ul	690.00	1	690.00	-	120	5.75	0.58
micropipetador automático 5 a 50 ul	690.00	1	690.00	-	120	5.75	0.58
microscópio de fluorescência invertido	23,990.00	1	23,990.00	2,399.00	120	179.93	39.98
microscópio óptico comum	1,081.08	1	1,081.08	-	120	9.01	0.90
mixer	572.09	1	572.09	-	120	4.77	0.48
pente da bomba de vácuo	317.32	1	317.32	-	12	26.44	-
pipetador manual	200.00	1	200.00	-	60	3.33	0.33
pipetador multicanal 20-200 ul 12 ponteiras	1,778.00	1	1,778.00	-	120	14.82	1.48
pipetador multicanal 20-200 ul 8 ponteiras	1,330.00	1	1,330.00	-	120	11.08	1.11
timer	173.00	2	346.00	-	120	2.88	0.29
tubo de plástico de 2,5 l para bomba de vácuo	39.00	1	39.00	-	60	0.65	-
tubos para centrifugação tipo Falcon®	0.85	16	13.60	-	60	0.23	-
Utensílios diversos de apoio ao teste							
autoclave de 60 l	8,594.40	1	8,594.40	859.44	60	128.92	28.65
armário de madeira	260.91	2	521.82	-	120	4.35	-
balão volumétrico de 2 l	77.00	1	77.00	-	60	1.28	-
balde para uso geral	19.90	1	19.90	-	60	0.33	-
bancada de aço	799.00	2	1,598.00	-	120	13.32	-
bancada de inox com pia	2,689.30	2	5,378.60	537.86	120	40.34	-
bancada de madeira/compensado	249.00	2	498.00	-	120	4.15	-
bandeja inox de uso geral	165.00	2	330.00	-	120	2.75	-
becker de 1000 ml	13.50	1	13.50	-	60	0.23	-
becker de 250 ml	5.40	1	5.40	-	60	0.09	-
becker de 500 ml	7.90	1	7.90	-	60	0.13	-
bomba de pressurização auxiliar externa para sistema de purificação de água Milli-Q	1,412.00	1	1,412.00	-	120	11.77	1.18

Direct-Q3 UV®							
cadeira giratória	339.00	2	678.00	-	120	5.65	-
calculadora	30.90	1	30.90	-	120	0.26	-
caneta esferográfica	0.47	1	0.47	-	12	0.04	-
caneta para escrever em plástico/vidro	1.74	1	1.74	-	48	0.04	-
carrinhos de aço	259.00	2	518.00	-	300	1.73	-
filtro de carvão para autoclave	390.00	1	390.00	-	12	32.50	-
filtro rápido para autoclave	230.00	1	230.00	-	12	19.17	-
geladeira	699.00	1	699.00	-	120	5.83	0.58
gelo reciclável	1.50	10	15.00	-	60	0.25	-
mesa de inox	960.00	1	960.00	-	120	8.00	-
módulo smartpak DQ3® de ultrapurificação de água para sistema de purificação de água Milli-Q Direct-Q3 UV®	1,564.92	1	1,564.92	-	4	391.23	-
pinça cirúrgica	16.00	1	16.00	-	60	0.27	-
potes de plástico com tampa para uso diverso	1.99	10	19.90	-	60	0.33	-
proveta de 100 ml	8.10	1	8.10	-	60	0.14	-
proveta de 1000 ml	38.20	1	38.20	-	60	0.64	-
proveta de 2000 ml	71.80	1	71.80	-	60	1.20	-
proveta de 500 ml	24.70	1	24.70	-	60	0.41	-
sistema de purificação de água Milli-Q Direct-Q3 UV®	10,755.00	1	10,755.00	1,075.50	120	80.66	17.93
termômetro de máxima e mínima para ambiente	49.50	1	49.50	-	120	0.41	0.04
tesoura cirúrgica	19.99	1	19.99	-	60	0.33	-
unidade filtrante millipak express® 40, 0,22µm de poro para sistema de purificação de água Milli-Q Direct-Q3 UV®	533.52	1	533.52	-	6	88.92	-
CUB							
edificações	595.00	40	23,800.00	2,380.00	300	71.40	7.93
					total	**1,704.69**	**168.05**

Operational staff workforce and Personal Protective Equipment (PPE) necessary to perform the Virus Isolation in Cell Culture (VICC), in Portuguese

Mão de obra e EPIs	Descrição	Preço (R$)	Preço unitário (R$)	n necessário	Preço total (valor inicial) (R$)
mão de obra direta: agente profissional médica veterinária	8 hs/dia, 40 hs/semana ou 160 hs/mês	4,809.10	4,809.10	1.00	4,809.10
mão de obra direta: técnico (agente de execução)	8 hs/dia, 40 hs/semana ou 160 hs/mês	2,003.78	2,003.78	1.00	2,003.78
EPI- aventais de tecido	unidade	49.00	49.00	10.00	490.00
EPI- óculos	unidade	3.99	3.99	2.00	7.98
				total	**7,310.86**

Electricity, water and gases necessary to perform the Virus Isolation in Cell Culture (VICC), in Portuguese

Item	Descrição	Preço (R$)	Preço unitário (R$)	n necessário	Preço total (valor inicial) (R$)
água	até 10 m^3/mês + 6,12/m^3 (excedente)	54.39		2 m^3/mês	54.39
luz	kWh= R$ 0.34805 x 4,320 Kw/mês		0.35	4,320 kw/mês	1512.00
aluguel cilindro de CO2 25 Kg	semestral	300.00	50.00	/mês	50.00
nitrogênio	litros	7.00	7.00	14 l/mês	98.00
				total	**1714.39**

Equipment licensing necessary to perform the Virus Isolation in Cell Culture (VICC), in Portuguese

Item	Descrição	Preço (R$)	preço unitário (R$)	n necessário	preço total (valor inicial) (R$)
licenciamento de equipamentos	contrato anual, pagamento mensal	3644.88	303.74	1.00	303.74
				total	**303.74**

Variable cost items necessary to perform the Virus Isolation in Cell Culture (VICC), in Portuguese

Variáveis	Descrição	Preço (R$)	Preço unitário (R$)	n necessário (máximo)	n/amostra	Preço por amostra (R$)
Reativos						
acetona	1 l	187.00	187.00	1,6 ml/placa	0.06 ml	0.01
álcool	1 l	7.35	7.35	1 l/semana	0.01 l	0.07
aminoácidos não essenciais	100 ml	58.00	0.58	40 ul/placa	1.5 ul	0.00
azul de Evans	10 g	213.00	0.02	0,0025 ml/placa	0.00	0.00
bicarbonato de sódio	1 Kg	174.00	174.00	0,088 g/placa	0.003 g	0.00
cilindro de CO2	25 Kg	150.00	6.00	8.33 Kg/mês	0.02 Kg	0.12
cloreto de sódio	1 Kg	149.00	149.00	0,41 g/placa	0.01 g	0.00
CN	1 ml	2.70	2.70	0,12 ml/placa	0.004 ml	0.09
conjugado	1 ml	0.00	0.00	6 ul/placa	0.00025 ml	0.00
CVS	1 ml	0.00	0.00	0,12 ml/placa	0.004 ml	0.00
gentamicina	250 mg	219.00	43.80	30 ul/placa	1.11 ul	0.05
glicerina	1 l	645.00	645.00	5 ml/placa	0.18 ml	0.12
hipoclorito	2,5 l	209.00	83.60	20 ml/mês	0.06 ml	0.01
meio mínimo essencial (Sigma®)	1 l	153.00	153.00	50 ml/placa	1.85 ml	0.28
NaH2PO4H2O	1 Kg	522.00	522.00	0,018 g/placa	0.00	0.00
NaHPO412H2O	1 Kg	569.00	569.00	0,13 g/placa	0.005 g	0.00
soro fetal bovino	500 ml	1265.00	2.53	2,2 ml/placa	0.08 ml	0.20
tripsina versene	500 ml	134.00	0.27	10 ml/placa	0.37 ml	0.10
Materiais diversos para os testes						
eppendorf®	500 unidades	46.20	0.09	200/ano	0.05	0.00
filtro para meio	unidade	37.65	37.65	1/20 placas	0.00	0.08
fita de autoclave	unidade	8.62	8.62	0.5 rolo de fita/mês	0.00	0.02
fita para medir pH	100 unidades	40.00	0.40	1/20 placas	0.00	0.00
garrafas de plástico de 25 ml para cultivo celular, com tampa e sem filtro	5 unidades	21.12	4.22	2/placa	0.07	0.30
lamínula para câmara de Neubauer®	100 unidades	6.60	0.07	1/placa	0.04	0.00
materiais para congelamento de células	20 ml/ano	53.29	2.67	1.67 ml/mês	0.01	0.01
papel filtro	100 unidades	25.75	0.26	2/placa	0.07	0.02

pipetas de plástico de 10 ml	10 unidades	18.00	1.80	2/placa	0.07	0.13
pipetas de plástico de 25 ml	10 unidades	31.80	3.18	2/placa	0.07	0.22
pipetas de plástico de 5 ml	10 unidades	17.30	1.73	2/placa	0.07	0.12
placas de 96 poços	10 unidades	114.09	11.40	1.00	0.04	0.46
ponteiras de 1 ml	1000 unidades	28.42	0.03	30/mês	0.09	0.00
ponteiras para pipetador automático 200 ul	96 unidades	11.90	0.12	500/placa	18.52	2.22
propé®	100 unidades	28.37	0.28	8/dia	0.66	0.18
touca	100 unidades	11.90	0.12	2/dia	0.16	0.02
tubos tipo falcon®	25 unidades	21.40	0.85	10/mês	0.03	0.03
folhas de ofício	500 unidades	19.90	0.04	1/placa	0.04	0.00
gaze	unidade	49.50	0.54	10 metros/mês	0.03	0.02
papel alumínio	unidade	3.50	3.50	1 rolo/2 meses	0.00	0.00
parafilm	unidade	98.01	98.01	1 rolo/ano	0.00	0.02
Custos associados à mão-de-obra						
EPI- luvas de látex	100 unidades	14.61	0.15	20/dia	1.70	0.26
EPI- máscara descartável	50 unidades	9.90	0.20	2/dia	0.16	0.03
					total	5.20

Depreciation and maintenance of durable and semi-durable goods necessary to perform the Mouse Inoculation Test (MIT), in Portuguese

Fixos	Valor unitário do bem	n necessário de bens	Valor total dos bens (preço inicial) (R$)	Valor residual (0% ou 10%) (R$)	Vida útil	Depreciação de bens duráveis e semiduráveis	Taxa de manutenção de bens duráveis e semiduráveis (0%, 10% ou 20%)
Equipamentos e materiais para os testes							
balança analítica	4041.60	1.00	4041.60	404.16	120.00	30.31	6.74
cabine de segurança biológica	56890.00	1.00	56890.00	5689.00	240.00	213.34	47.41
caixa para câmara de CO2	978.04	1.00	978.04	0.00	60.00	16.30	0.00
lâmpada para cabine de segurança biológica	532.72	1.00	532.72	0.00	11.00	48.43	0.00
mangueira para câmara de CO2	73.35	1.00	73.35	0.00	60.00	1.22	0.00
manômetro para cilindro de CO2	130.68	1.00	130.68	0.00	120.00	1.09	0.11
pinças	7.05	3.00	21.15	0.00	60.00	0.35	0.00
suporte de tela e madeira	9.90	1.00	9.90	0.00	60.00	0.17	0.00
Utensílios diversos de apoio ao teste							
autoclave de 303 l	47412.02	1.00	47412.02	4741.20	60.00	711.18	158.04
armário de madeira	260.91	1.00	260.91	0.00	120.00	2.17	0.00
balde grande para ração	75.00	1.00	75.00	0.00	60.00	1.25	0.00
balde para uso geral	19.90	3.00	59.70	0.00	60.00	1.00	0.00
bancada de inox com pia	2689.30	2.00	5378.60	537.86	120.00	40.34	0.00
cadeira giratória	339.90	3.00	1019.70	0.00	120.00	8.50	0.00
calculadora	30.90	1.00	30.90	0.00	120.00	0.26	0.00
caneta esferográfica	0.48	1.00	0.48	0.00	12.00	0.04	0.00
caneta para escrever em plástico/vidro	1.74	1.00	1.74	0.00	48.00	0.04	0.00
carrinho de ferro para carregar balde de ração	370.00	1.00	370.00	0.00	300.00	1.23	0.00
carrinho de ferro para cubas de maravalha	500.00	1.00	500.00	0.00	300.00	1.67	0.00
carrinhos de aço	259.00	2.00	518.00	0.00	300.00	1.73	0.00
cubas grandes	120.00	1.00	120.00	0.00	60.00	2.00	0.00
escada de material inoxidável	79.90	1.00	79.90	0.00	300.00	0.27	0.00

escova espiral para lavagem de garrafas	7.95	2.00	15.90	0.00	6.00	2.65	0.00
espátula	10.90	1.00	10.90	0.00	60.00	0.18	0.00
esponja dupla face	0.65	2.00	1.30	0.00	1.00	1.30	0.00
estantes de ferro de uso geral	115.00	5.00	575.00	0.00	300.00	1.92	0.00
fichário	25.50	1.00	25.50	0.00	120.00	0.21	0.00
filtro de água para torneira	110.00	4.00	440.00	0.00	6.00	73.33	0.00
filtro de carvão para autoclave	390.00	1.00	390.00	0.00	12.00	32.50	0.00
filtro rápido para autoclave	230.00	1.00	230.00	0.00	12.00	19.17	0.00
mesa de inox	960.00	3.00	2880.00	288.00	120.00	21.60	0.00
panos de chão	1.49	16.00	23.84	0.00	60.00	0.40	0.00
potes de plástico com tampa para uso diverso	1.99	10.00	19.90	0.00	60.00	0.33	0.00
sistema de climatização	83308.00	2.00	166616.00	16661.60	300.00	499.85	111.08
termômetro de máxima e mínima para ambiente	49.50	1.00	49.50	0.00	120.00	0.41	0.04
CUB							
edificações do biotério	595.00	85.00	50575.00	5057.50	300.00	151.73	16.86
					total	**1888.45**	**323.41**

Operational staff workforce and Personal Protective Equipment (PPE) necessary to perform the Mouse Inoculation Test (MIT), in Portuguese

Mão de obra e EPIs	Descrição	Preço (R$)	Preço unitário (R$)	n necessário	Preço total (valor inicial) (R$)
mão de obra direta: agente profissional médica veterinária	8 hs/dia, 40 hs/semana ou 160 hs/mês	4809.10	4809.10	1.00	4809.10
mão de obra direta: técnico (agente de execução)	8 hs/dia, 40 hs/semana ou 160 hs/mês	2003.78	2003.78	1.00	2003.78
mão de obra indireta: médica veterinária RT	1 h/dia, 5 hs/semana	9220.80	230.52	1.00	1152.60
EPI- avental de plástico	unidade	30.00	30.00	3.00	90.00
EPI- aventais de tecido	unidade	49.00	49.00	15.00	735.00
EPI- óculos	unidade	3.99	3.99	2.00	7.98
				total	**8798.46**

Electricity, water and gases necessary to perform the Mouse Inoculation Test (MIT), in Portuguese

Item	Descrição	Preço (R$)	Preço unitário (R$)	n necessário	Preço total (valor inicial) (R$)
água	até 10 m³/mês + 6,12/m^3 (excedente)	54.39		8 m^3/mês	54.39
luz	kWh= R$ 0,34267 x 6,516 kw/mês		0.35	6,516 kw/mês	2280.60
aluguel cilindro de CO2 6 Kg	semestral	150.00	25.00	/mês	25.00
				total	**2359.99**

Equipment licensing necessary to perform the Mouse Inoculation Test (MIT), in Portuguese

Item	descrição	preço	preço unitário	n necessário	preço total (valor inicial)
licenciamento de equipamentos	contrato anual, pagamento mensal	1560.24	130.02	1.00	130.02
				total	**130.02**

Variable cost items necessary to perform the Mouse Inoculation Test (MIT), in Portuguese

Variáveis	Descrição	Preço (R$)	Preço unitário (R$)	n necessário (máximo)	n/amostra	Preço por amostra (R$)
Reativos						
álcool	1 l	7.35	7.35	4 l/semana	0.08 l	0.59
cilindro de CO2	6 Kg	50.00	8.33	0,5 Kg/mês	0.0025 Kg	0.17
cloro 10%	5 l	9.80	1.96	1 l/semana	0.02 l	0.04
detergente alcalino	5 l	19.65	3.93	2 l/semana	0.04 l	0.16
Materiais diversos para os testes						
agulha	100 unidades	12.00	0.12	1 /animal	7.00	0.84
algodão	1 kg	32.00	32.00	1 g/animal ou 1 Kg/mês	0.005 Kg	0.16
camundongo	unidade	5.00	5.00	7 /amostra	7.00	35.00
cepilho de madeira	1 Kg	8.50	8.50	350 g/amostra	350 g	2.98
fita de autoclave	unidade	8.62	8.62	1 rolo de fita/mês	0.01	0.04
propé®	100 unidades	28.37	0.28	12/dia	1.70	0.48
ração	20 Kg	80.00	4.00	1 Kg/amostra	1 Kg	4.00
sacos de plástico para autoclave	20 unidades	22.26	1.11	5/dia	0.71	0.79
saquinhos de plástico (comum)	2000 unidades	33.60	0.02	10/dia	1.43	0.03

seringa	unidade	0.40	0.40	1/dia	0.14	0.06
touca	100 unidades	11.90	0.12	3/dia	0.43	0.05
fichas de papel cartolina	100 unidades	3.30	0.03	1/amostra	1.00	0.03
folhas de ofício	500 unidades	19.90	0.04	1/semana	0.02	0.00
papel alumínio	unidade	3.50	3.50	1 rolo/2 meses	0.00	0.01
papel toalha	4 embalagens	18.67	4.67	4 fardos/mês	0.02	0.09
plástico para etiquetas(envelope)	50 unidades	7.00	0.14	1/amostra	1.00	0.14
Custos associados à mão-de-obra						
EPI- luvas amarelas	2 unidades	2.59	1.30	2/mês	0.01	0.01
EPI- luvas de látex	100 unidades	14.61	0.15	12/dia	1.70	0.26
EPI- máscara com filtro	unidade	16.20	16.20	1/2 meses	0.00	0.04
EPI- máscara descartável	50 unidades	9.90	0.20	3/dia	0.43	0.09
					total	**46.04**

APPENDIX XIII

Ato da Diretoria Executiva 28/2014 referente à Chamada de Projetos 24/2012-Programa Universal / Pesquisa Básica e Aplicada

***Resultado final de pesquisa básica e aplicada*, available at:**

http://www.fappr.pr.gov.br/arquivos/File/diretoria/atos2014/Ato028_14_CP24_12_ResultadoPBA.pdf

FUNDAÇÃO ARAUCÁRIA
Apoio ao Desenvolvimento Científico e Tecnológico do Paraná

UEL	38144	Meta-análise de experimentos em fitopatologia visando produção agrícola sustentável	Marcelo Giovanetti Canteri
UFPR	38615	Subsídios à Implantação de Alternativas Validadas para Substituir o Uso de Animais de Laboratório: o diagnóstico da raiva como modelo	Carla Forte Maiolino Molento
MODALIDADE C			
IES	**PROT.**	**TITULO DO PROJETO**	**COORDENADOR**
CIÊNCIAS AGRÁRIAS			
UEPG	35856	Identificação e análise de genes diferencialmente expressos durante o amadurecimento dos frutos de melão climatério e não climatério	Ricardo Antonio Ayub
UEL	36799	Desenvolvimento de materiais biodegradáveis a base de amido e álcool polivinílico (PVOH)	Fabio Yamashita
CIÊNCIAS BIOLÓGICAS			
UFPR	38936	Estudo metabolômico de linhagens de melanoma humano e avaliação das vias p53, GAMT e PKC em linhagens de melanócitos dependentes de PMA para crescimento.	Guilherme Lanzi Sassaki
CIÊNCIAS EXATAS E DA TERRA			
UFPR	39283	Síntese e caracterização de nanoestruturas Au/PEDOT estabilizadas por polímeros naturais e sua aplicação em dispositivos eletroquímicos de alto desempenho	Marcio Eduardo Vidotti Miyata
UFPR	39864	Produção de biodiesel e triacetina por interesterificação química do óleo de soja com acetato de etila utilizando materiais lamelares como catalisadores heterogêneos	Luiz Pereira Ramos
CIÊNCIAS DA SAÚDE			
UEM	35936	Investigação da atividade terapêutica e toxicológica de Stryphnodendron adstringens (Mart.) Coville (Barbatimão) aplicada às mulheres para o tratamento de candidíase vulvo-vaginal	Celso Vataru Nakamura
UEM	39310	Avaliação do possível impacto da radiação gama, usada em tratamento de câncer de cabeça e pescoço, sobre a virulência de leveduras do gênero Candida	Terezinha Inez Estivalet Svidzinski
CIÊNCIAS SOCIAIS APLICADAS			
PUC PR	35835	Concorrência e propriedade industrial no mercado de bebidas frias: inovação, desenvolvimento e eficiência	Marcia Carla Pereira Ribeiro
ENGENHARIAS			
UTFPR	35849	Estruturas fotorrefrativas e guias de onda oticamente induzidos	Hypolito José Kalinowski
LINGUÍSTICA, LETRAS E ARTES			
UEL	39738	(Educ)ação de professores de línguas: trabalho e desenvolvimento na práxis docente	Vera Lúcia Lopes Cristovão
MULTIDISCIPLINAR			
UFPR	38196	Emprego de um novo larvicida de baixa toxicidade e de mistura de compostos naturais voláteis com atividade adulticida visando o controle biorracional dos vetores da dengue e malária (mosquitos Aedes aegypti e Anopheles spp. (Diptera, Culicidae)	Mario Antônio Navarro da Silva

Curitiba, 15 de abril de 2014.

ANNEX 1

The University of British Columbia
Office of Research Services
Behavioural Research Ethics Board
Suite 102, 6190 Agronomy Road, Vancouver, B.C. V6T 1Z3

CERTIFICATE OF APPROVAL - MINIMAL RISK AMENDMENT

PRINCIPAL INVESTIGATOR:	DEPARTMENT:	UBC BREB NUMBER:
Peter A. Danielson	UBC/College for Interdisciplinary Studies/Applied Ethics	H11-02849

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT:

Institution	Site
UBC	Vancouver (excludes UBC Hospital)

CO-INVESTIGATOR(S):
Dan M. Weary
Vanessa Carli Bones

SPONSORING AGENCIES:
Teaching and Learning Enhancement Fund - "Computer Enhanced Experimental Applied Ethics Education (CEE-Ethics)"

PROJECT TITLE:
Your Views on the Use of Animals for Rabies Diagnosis

Expiry Date - Approval of an amendment does not change the expiry date on the current UBC BREB approval of this study. An application for renewal is required on or before: November 18, 2012

AMENDMENT(S): | **AMENDMENT APPROVAL DATE: January 10, 2012**

Document Name	Version	Date
Consent Forms:		
rabies diagnosis Portuguese consent	1	November 26, 2011
rabies diagnosis consent	3	November 26, 2011
CEE-Ethics Portuguese Consent form	N/A	November 25, 2011
Advertisements:		
rabies diagnosis Portuguese recruitment	N/A	November 25, 2011
Questionnaire, Questionnaire Cover Letter, Tests:		
rabies diagnosis study Portuguese content page 2	N/A	November 27, 2011
rabies diagnosis study content page 1	2	November 26, 2011
rabies diagnosis study welcoming page - English and Portuguese	N/A	November 25, 2011
rabies diagnosis study Portuguese content page 1	N/A	November 27, 2011
rabies diagnosis study Portuguese content page 3	N/A	November 27, 2011
rabies diagnosis study Portuguese content page 4	N/A	November 27, 2011
rabies diagnosis study Portuguese content page 5	N/A	November 27, 2011
Other:		
The survey will appear as http://your-views.org/D7/rabies_diagnosis The content of the survey portion of the site is provided under 9.6.		

The amendment(s) and the document(s) listed above have been reviewed and the procedures were found to be acceptable on ethical grounds for research involving human subjects.

This study has been approved either by the full Behavioural REB or by an authorized delegated reviewer