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https://doi.org/10.22239/2317-269X.02283

Study of the difficulties encountered by Brazilian laboratories in implementing alternative methods to animal use: assessment of Ocular Irritation and the Bovine Corneal Opacity and Permeability (BCOP) test

Estudo das dificuldades encontradas por laboratórios brasileiros na implementação de métodos alternativos ao uso de animais: avaliação de irritação ocular e o teste de Permeabilidade e Opacidade da Córnea Bovina (BCOP)

Izabela Gimenes[I,II,*]

Octavio Augusto França Presgrave[I,III]

Marcelo Salabert Gonzalez[I,IV]

Gutemberg Gomes Alves[I,V]

[I] Programa de Pós-Graduação em Ciências e Biotecnologia, Instituto de Biologia, *Campus* Gragoatá, Universidade Federal Fluminense, Niterói, RJ, Brasil

[II] Laboratório de Farmacologia, Instituto Nacional de Controle de Qualidade em Saúde, Fundação Oswaldo Cruz (INCQS/Fiocruz), Rio de Janeiro, RJ, Brasil

[III] Centro Brasileiro de Validação de Métodos Alternativos (BraCVAM), Fundação Oswaldo Cruz (Fiocruz), Rio de Janeiro, RJ, Brasil

[IV] Laboratório de Biologia de Insetos, Departamento de Biologia Geral, Instituto de Biologia, Universidade Federal Fluminense, Niterói, RJ, Brasil

[V] Departamento de Biologia Celular e Molecular, Instituto de Biologia, Universidade Federal Fluminense, Niterói, RJ, Brasil

* **E-mail:** izabela.gimenes@fiocruz.br

Received: Nov 17, 2023
Approved: Sep 02, 2024

How to cite: Gimenes I, Presgrave OAF, Gonzalez MS, Alves GG. Study of the difficulties encountered by Brazilian laboratories in implementing alternative methods to animal use: assessment of Ocular Irritation and the Bovine Corneal Opacity and Permeability (BCOP) test. Vigil Sanit Debate, Rio de Janeiro, 2024, v.12: e02283. https://doi.org/10.22239/2317-269X.02283

ABSTRACT

Introduction: Ensuring the safety of products causing ocular irritation is crucial. The Bovine Corneal Opacity and Permeability (BCOP) test is an accepted alternative method for ocular irritation assessment in regulatory contexts. **Objective:** This study aimed to identify the main challenges and technical limitations faced by professionals when implementing alternative methods, particularly for ocular irritation assessment. **Method:** A qualitative research approach was employed using questionnaires (n = 22 respondents) and interviews (n = 7), followed by discourse analysis. **Results:** Findings reveal that 71% of alternative method practitioners encounter significant difficulties, primarily due to high validation/implementation costs (62%), driven by consumable expenses. Limited access to training (62%) and importation of reagents and equipment (43%) were also noted. Key BCOP limitations include restricted access to bovine eyeballs (50%), less precise risk classification (43%), and substantial eyeball waste (29%) due to damage. Interviews supported these conclusions, highlighting challenges such as travel to slaughterhouses, insufficient technical details in the OECD Guide, the need for additional support materials, and a lack of experienced professionals. These results underscore the need for expanded training opportunities for test implementation. The high cost of imported materials indicates greater validation expenses in Brazil than in other countries. **Conclusions:** Addressing these challenges requires an open innovation environment, fostering collaboration among the Triple Helix (Companies, Scientific and Technological Institutions, and Government).

KEYWORDS: Alternative Methods; Toxicological Tests; Eye Irritation; BCOP, Interview

RESUMO

Introdução: Garantir a segurança dos produtos que causam irritação ocular é crucial. O Teste de Opacidade e Permeabilidade da Córnea Bovina (BCOP) é um método alternativo de irritação ocular aceito para fins regulatórios. **Objetivo:** Este estudo visou identificar os principais desafios e limitações técnicas enfrentadas por profissionais na implementação de métodos alternativos, particularmente para avaliação de irritação ocular. **Método:** Uma pesquisa qualitativa foi realizada a partir de questionários (n = 22 respondentes) e entrevistas (n = 7) seguidas por análise de discurso. **Resultados:** Os resultados revelam que 71% dos executores de métodos alternativos enfrentam dificuldades significativas, principalmente devido aos elevados custos de validação/implementação (62%) motivados por despesas com consumíveis. O acesso limitado à formação (62%) e a importação

de reagentes e equipamentos (43%) também foram apontados. As principais limitações do BCOP incluem acesso limitado a globos oculares bovinos (50%), classificação de risco menos precisa (43%) e desperdício substancial de globos oculares (29%) devido a danos. As entrevistas corroboraram estas conclusões, destacando desafios como deslocamento aos matadouros, detalhes técnicos insuficientes no Guia da OCDE, a necessidade de materiais de apoio adicionais e a falta de profissionais experientes. Estes resultados ressaltam a necessidade de maior oferta de treinamentos para implementação de testes. Os altos custos de materiais importados apontam maiores gastos com validação no Brasil em comparação com outros países. **Conclusões:** Enfrentar estes desafios demanda um ambiente de inovação aberto, promovendo a colaboração entre a Tríplice Hélice (Empresas, Instituições Científicas e Tecnológicas e Governo).

PALAVRAS-CHAVE: Métodos Alternativos; Avaliação Toxicológica; Irritação Ocular; BCOP; Entrevista

INTRODUCTION

Brazil ranks fourth in the consumption of personal hygiene, perfumery, and cosmetics products, generating approximately U$ 22.9 billion a year in the world economy[1]. Of this, 15% goes on research, development, communication, and the launch of new products[2]. The easy availability of these items in various sales channels has contributed to the growth of the market, which is increasingly impacted by male consumers, as well as generations Z and *millenials*.[3]

Considering that these products will be used daily by millions of consumers, as indicated by the economic indices presented, guaranteeing their safe use before they are marketed becomes an important public health issue and one to which health surveillance should pay attention. These products can cause adverse reactions depending on their composition, quantity, exposure time, and the individual sensitivity of the user[4]. Recently, the Brazilian National Health Surveillance Agency (Anvisa) notified the precautionary banning of a hair ointment due to the presence of undesirable effects on consumers such as: temporary blindness (temporary loss of vision), severe burning in the eyes, intense tearing, itching, redness, eye swelling, and headache[5]. Incidents like this reinforce the need for regulatory bodies to be in line with national and international methodologies that assess the safety of products with the potential for eye irritation and corrosion. For many years, the methodology used to evaluate these products was the Draize test, which exposes the eyes of rabbits to possible toxic agents, generating ethical concerns due to the suffering caused to the animals[6]. In this way, scientifically valid questions that replace, reduce, or refine animal use have led to a search for alternative methods to make safe ingredients and products available through the execution of toxicological tests, which are mandatory for the country's scientific and technological development[6,7].

Over the last few decades, Brazil has devoted a lot of effort to this area and has made a commitment to: i) formulating rules on the humane use of animals for teaching and scientific research purposes, through the creation of the National Council for the Control of Animal Experimentation (Concea)[8,9]; and ii) to promote the development and validation of new techniques in Brazil and the certification of alternative methods to the use of animals, with the establishment of the National Network of Alternative Methods (Renama)[10], as well as the creation of a Regional Platform for Alternatives to Animal Experimentation Methods (PReMASUL), to promote the creation of a laboratory infrastructure and specialized human resources capable of implementing alternative methods within the scope of the Southern Common Market (Mercosur).

Concea recognizes that a validated alternative method is one with international regulatory acceptance and establishes a deadline of five years for them to come into force as a mandatory replacement for the original method from the date of publication of each corresponding Normative Resolution (NR)[11]. Anvisa, through Collegiate Board Resolution (RDC) No. 35 of August 11, 2015, accepts the use of alternative methods of animal experimentation recognized in Brazil by Concea[12]. This act gave regulatory weight to the methods approved by Concea, impacting the entire production sector regulated by Anvisa. A crucial aspect of the regulatory issue is the participation of the Organization for Economic Co-operation and Development (OECD), an intergovernmental organization that represents more than 30 countries. Its prerogatives include policy coordination and harmonization, discussion of issues of mutual concern and cooperation to tackle international problems. The guidelines for carrying out safety tests are available on the organization's website.[13]

In 2023, the ban on the use of vertebrate animals in scientific research, development, and control of personal care products, perfumes, and cosmetics that contain in their formulation ingredients or compounds with scientifically proven safety and efficacy in Brazil, represented a milestone in the advancement in ethical terms and scientific standards in the area. This measure triggered the mandatory requirement to use alternative methods in this area, converging with international standards in the sector.[14]

Concea currently recognizes 41 alternative methods, grouped into 15 outcomes for mandatory replacement, seven of which are for assessing the potential for eye irritation and corrosion (Chart 1).

Each of these methods has their own characteristics. The Bovine Cornea Opacity and Permeability Test (BCOP) and the Isolated Chicken Eye Test (ICE) use freshly slaughtered animal eyes preserved *in vitro*, with the advantage of using histology to increase their sensitivity[24]. The BCOP applies test substances to a bovine cornea mounted on a holder, assessing opacity and permeability using fluorescein. Opacity is measured by the transmission of

Chart 1. Methods recognized by Concea for assessing the potential for eye irritation and corrosion.

Normative Resolution No. 18/2014[15]	OECD 437 - Bovine Cornea Opacity and Permeability Test (BCOP)[16]
	OECD 438 - Isolated Chicken Eye Test (ICE)[17]
	OECD TG 460 - Fluorescein Leakage Test[18]
Normative Resolution No. 31/2016[19]	OECD 491 - *In vitro* Short Term Exposure (STE)[20]
	OECD 492 - Reconstructed Human Cornea-like Epithelium (RhCE)[21]
Normative Resolution No. 56/2022[14]	OECD TG 494 - Vitrigel eye irritation test[22]
	OECD TG 496 - *In vitro* macromolecular test[23]

Source: Prepared by the authors, 2023.

light through the cornea, while permeability is measured by the amount of fluorescein that passes through the cornea, detected in the posterior chamber of the holder with the help of a visible light spectrophotometer[16]. These measurements calculate the *in vitro* irritancy score (IVIS) which is used to classify the degree of *in vitro* eye irritation of the chemical tested. The method has different protocols for liquids/surfactants (10 min) and non-surfactant solids (4 h). It is suitable for detecting moderate to severe irritants, but not mild ones[16,24]. The ICE test, on the other hand, observes damage by applying the test substance to the chicken eye, assessing opacity, thickness, and fluorescein retention, with easily available eyes[25]. However, surfactants and alcohols can cause false negatives and positives.[17]

Monolayer cultured cell assays are simple and short but do not reflect the three-dimensional microenvironment of real tissues[24]. The *in vitro* Short Term Exposure (STE) assesses cytotoxicity in SIRC cells using MTT, useful for identifying severe irritants and non-irritants, but not recommended for Category 2[20]. The Fluorescein Leakage Test (FL) evaluates cell function in canine kidney epithelial cells (MDCK), with limitations for colored, viscous substances and certain cell lines[18]. Three-dimensional models such as the Reconstructed Human Cornea-like Epithelium (RhCE) mimic functional tissue, assessing primary irritation and cytotoxicity. However, they are fragile and limited to reproducing only the epithelial layer, not addressing systemic effects or penetration into the stroma/endothelium[24]. Although RhCE has been adopted as an independent *in vitro* method to discriminate between the three categories defined by the United Nations (UN) Globally Harmonized System of Classification and Labelling of Chemicals (GHS): Category 1 (Cat. 1) for "serious eye damage"; Category 2 (Cat. 2) for "eye irritation" and No Category (No Cat.) for chemicals "not requiring eye irritation".) for chemicals "not requiring classification and labeling" for eye irritation or serious eye damage[26], the data generated has been proposed to be used together, as the "Top-Down" or "Bottom-Up" approaches, which combine different *in vitro* methods to cover the necessary irritation potential.[27,28]

The Vitrigel® Ocular Irritation Test uses human corneal epithelial models on a collagen membrane, measuring epithelial barrier function by transepithelial electrical resistance (TEER). It is not validated for substances of unknown or complex composition, biological materials, mixtures, gases, and aerosols[22]. Finally, the *in vitro* macromolecular test identifies chemicals that cause serious eye damage or do not require an eye irritation classification, through an acellular biochemical system, measuring the turbidity caused by the disintegration of the macromolecular matrix.[23]

Despite the regulatory acceptance of the seven methods for predicting ocular irritation responses in the Draize Test, we recognize the need for an approach based on human biology that provides support to guarantee product safety. Given that there are multiple irritation mechanisms involved, it is crucial to obtain data related to vascularization, opacity/permeability, and cytotoxicity[29]. A single *in vitro* test is not sufficient for a comprehensive assessment of different chemical classes[6], which is driving efforts towards New Approach Methodologies (NAMs).[27,30,31,32]

In this context, the OECD's international directives guide decision-making for the classification and labeling of products in serious eye damage/eye irritation testing and evaluation using data based on a set, as well as information sources, physicochemical properties, *in silico* and read-across predictions of chemical analogues, within Integrated Approaches to Testing and Evaluation (IATA) (IATA n° 263[27]) or Defined Approaches (DAs) (OECD TG 467[34]). BCOP is used as an initial test within a top-down assessment strategy to identify chemicals that cause serious eye damage[26,27,28], as well as for chemicals not classified for eye irritation or serious eye damage (UN GHS No Category - NC, uncategorized) and has therefore been endorsed as scientifically valid for both purposes.[27]

When we look at the significant volume of more than 80 billion animals slaughtered internationally each year for meat consumption, among them 302 million cattle[25] we can see a valuable opportunity for BCOP testing, where eyes are often discarded after slaughter because they are not normally marketable in Brazil[36,37]. Considering the example of the National Institute for Quality Control in Health (INCQS), an official laboratory that is part of Anvisa's National Network of Health Surveillance Laboratories (RNLVISA) and is the national reference for the network[38], the distance from the official slaughterhouse where the eyes are collected to their arrival at the Institute becomes a major limitation in the application of BCOP, as they are on average 140 km apart (132 to 150 km), since the journey takes up almost the entire time limit for the corneas to be used. Therefore, there is a clear need to verify the impact of the distance between the official slaughterhouses and the test centers, as the interval between collection and the start of the test must be minimized and demonstrated so as not to compromise the results[16]. Another limiting factor to be considered is the difficulty in finding a slaughterhouse willing to cooperate technically by donating or selling these eyeballs. In addition, technical difficulties relating to the lengthy execution of the method can take from 8 to 12 hours.

Currently, there are two defined approaches (DAs) adopted by the OECD for the identification of ocular risks from liquids (OECD TG 467[33]). Both use the Bovine Corneal Opacity and Permeability (BCOP) method with a laser light-based opacimeter (LLBO), in accordance with OECD TG 437[16], in a combination of strategies. DAL-1 uses the Reconstructed Human Cornea-like Epithelium (RhCE) method, which can be the EpiOcular™ Irritation Test or the Human Corneal Epithelium (HCE) Irritation Test (SkinEthic™), according to OECD TG 492[21], and is aimed at pure non-surfactant liquids[33]. On the other hand, DL-2 combines the BCOP LLBO with the in vitro Short Term Exposure (STE) test, in accordance with OECD TG 491[20], and is applicable to pure non-surfactant liquids, liquids and solids dissolved in water.[33]

A recent study aimed to develop a defined approach for identifying the eye risks of solid chemicals according to the three UN GHS categories (Cat. 1, Cat. 2, No Category): the DAS. It has been demonstrated in the context of the IATA concept that the DAS is a reliable defined approach for the assessment of eye risks from solids according to the UN GHS. In step 1, the SkinEthic™ HCE EIT test method is used to identify "No Category" (No Cat.); in step 2, the BCOP LLBO is used to identify "Category 1" (Cat. 1). This approach can be considered a complete non-animal substitute (NAM) for the Draize in vivo test.[34]

BCOP offers an *in vitro* approach to assessing the irritation and potential ocular corrosion of chemical substances and products, in line with the principles of replacement, reduction, and refinement in the use of animals in research, known as the 3Rs. However, literature lacks exploratory studies that identify problems and sustainable technical alternatives to overcome these difficulties, thus allowing the widespread adoption of this method as a process innovation for obtaining biological material. This is of particular relevance to public health, where toxicological tests must be carried out in order to guarantee consumer safety and the efficacy of products.

In this context, this study aims to map the difficulties encountered by Brazilian laboratories in implementing alternative methods to the use of animals, with a special focus on the outcome of ocular irritation through BCOP, by means of a qualitative analysis among executors of alternative methods, capable of identifying gaps in knowledge and obtaining insights to support future research and decision-making.

MATERIALS AND METHODS

This study is an exploratory and descriptive field study with a qualitative and quantitative approach. This research is part of a project approved by the Research Ethics Committee (CEP) of the Fluminense Federal University (UFF), opinion No. 3.753.708. The approach included the analysis of data collected from professionals in the field of alternative methods, based on questionnaires and interviews, initially raising a general picture of the difficulties in implementing them and then provoking a discussion focused on the implementation of the BCOP method.

The study was reported according to the consolidated criteria for reporting qualitative research (COREQ), which consists of a 32-item checklist for interviews and focus groups, covering methodology for data collection in qualitative health research.[39,40]

Research team

The interviews and questionnaires were conducted from January 2019 to December 2021 by a coded interviewer (IGL), according to the methodology of Tong et al.[40]. The interviewer was female, technically qualified, and had more than 15 years' experience in carrying out alternative methods to the use of animals. Prior to the start of the study, a prior relationship was established with the research participants, in which the interviewer was personally introduced, the study was presented, and a statement of personal interests was made, identifying the objectives and reasons for carrying out the research.

Study design

Participant selection and sample size

Volunteer participants were selected via a list received through prior contact with the coordination of Renama/Ministry of Science, Technology and Innovation (MCTI) and the researchers' personal contact lists. Participants were approached in person, at scientific events, by telephone, or by means of an electronic form made available via an e-mail link.

Each participant signed the Informed Consent Form (ICF) in person or electronically. They were shown the objectives and all the main information contained in the ICF, including the fact that non-participation does not imply any harm to the functional activity of the team members, and that the use of the project data anonymously will only be for academic purposes and not for professional evaluation.

A total of 51 volunteers were approached, including executors of alternative methods to the use of animals in Brazil, managers, professionals, and students from academic laboratories, research centers, and other laboratories belonging to the public and/or private network that are on the access list of Renama member laboratories. Professionals were excluded if, despite working in implementing institutions, they had no direct or indirect experience in implementing alternative methods. In the end, 22 participants answered the questionnaire. For the interview stage, potential participants were recruited during specialized scientific events in the field of alternative methods, and seven participants were selected, aged between 18 and 75 and of both sexes, who had already had previous contact with the BCOP methodology.

Study procedure

The study was carried out in two stages. The first involved administering questionnaires in person and online, via a link provided by e-mail to participants who signed the informed consent form. The questionnaire consisted of closed questions, from which frequencies of responses were calculated, and open questions which were coded and analyzed. For data collection in the first stage, the authors provided questionnaire guides for conducting the study

(supplementary material 1). The questionnaires were initially tested among 10 members of a public health laboratory to check the accuracy of the questions and the collection of information.

The second stage involved a purposive sample of seven participants who were invited for individual or group interviews comprising four to six participants, who signed a second ICF for the interview. The aim was to validate the new knowledge acquired during the first stage of the questionnaires. A guide instrument was used to conduct the semi-structured interviews (supplementary material 2). The interviews were carried out using online videoconferencing tools or in person, during three scientific events in the field of toxicology and alternative methods, when representatives of Renama's member laboratories and other institutions of interest took part.

For the second stage of data collection, the interviews were conducted by a member of the research group. A notebook was used for field notes in which the interview responses were transcribed using audio recording, coding by profession and number, and discourse analysis, with their permission, and were not returned to the participants for further comments. The interviews lasted approximately 10 to 20 minutes. Data saturation was discussed to indicate that the objective of an in-depth understanding of the phenomenon studied had been achieved with the selected sample. All documents were archived in a secure place with restricted access to members of the research team.

Based on the answers, the items were systematically organized for a qualitative discourse analysis, where the statements that highlight the issues were transcribed in quotation marks. Secondary themes were described to clarify the results and discussion.

Data analysis and results

The results of the questionnaires were analyzed qualitatively and quantitatively. The data was then tabulated in a Microsoft Excel™ spreadsheet by category, and the results were subjected to independent verification by a specialist researcher (GAG) who had no previous knowledge or professional links with the research participants. Only the frequency of numerical responses was calculated, no statistical data was processed, and no software was used to transcribe the responses.

The transcriptions of the interviews were carried out by the authors manually without the use of specialized software, and went through two different approaches in the research:

a. A discourse analysis aimed to examine the forms of linguistic expression used, in which similar statements and ideas were grouped into categories to illustrate the themes (thematic analysis), and the findings and quotes from the participants were coded and identified by profession code followed by number, similar to previous work by Timoteo et al.[40] and Orri et al.[41,42]. The data is summarized in a synoptic table (Chart 2).

b. A content analysis, with the aim of systematically organizing the data into a structured format. The results were synthesized into themes, which included: limited availability of eyeballs at the slaughterhouse; waste of eyeballs; difficulty in classifying the method; travel from the laboratory to the slaughterhouse; technical problems in execution; difficulties in implementing the methodology; use of the OECD guide; cost, investment and legislation; need for and access to more courses and training; dissemination of alternative methods in undergraduate and postgraduate courses. Secondary themes were described for clarity of results and discussion.

RESULTS

Analysis of the questionnaires

In the first stage of the study, of the 51 researchers contacted on the Renama list to apply the questionnaires, 22 respondents were included, of whom 20 (90.9%) perform the methods, one participant (4.5%) has already worked and one does not perform but outsources the service (4.5%) with alternative methods to the use of animals. The collection dates were recorded in the documents. No participants dropped out of the questionnaires. The number of respondents to each question is shown in the figure legend.

When asked about the method used for the eye irritation and corrosion endpoint, we obtained a total of 17 respondents, a third of whom, i.e. six participants (35.3%), indicated that they carry out the BCOP test (OECD TG 437), while 58.8% (10 respondents) carry out the *in vitro* short-term test (STE) (OECD TG 491) to assess the potential for eye irritation (Figure 1).

The results of the questionnaires, answered by eight participants, revealed that, in terms of the difficulties encountered in carrying out the BCOP test, the greatest was the lack of availability of slaughterhouses to supply bovine eyes for the test (four respondents, or 50%), followed by the large amount of eyeballs wasted due to scratches and other damage (three respondents, or 37.5%). In addition, difficulties were pointed out such as the method not providing as assertive a classification as the *in vivo* method (three respondents, or 37.5%), the journey from the laboratory to the slaughterhouse (25%) and the need for histopathological assessment (25%) (Figure 2).

When asked how long it takes for the executors to travel between collecting the bovine eyes and arriving at the laboratory, seven respondents revealed that the majority (three respondents, or 42.9%) travel between 2 and 3 hours.

With a view to finding opportunities for improvement to meet the needs of those carrying out alternative methods and understanding their main difficulties in carrying out tests, all 22 participants answered this question. The majority pointed to the high cost of the validation process (15 respondents or 68.0%) followed by the high cost of materials (13 respondents or 59.1%) and the purchase of equipment (nine respondents or 40.9%). Opportunities related to teaching were pointed out by 59.1%, who feel the need for more courses and training in the area

Chart 2. Summary table with the content of the interview transcripts.

Training	Is the OECD guide self-explanatory?	Difficulties in using the OECD Guides	Other difficulties	Where does the demand come from?	How important is a survey of laboratories to disseminate the methods?	Any other suggestions or comments?
Biologist 1	“No. I think it’s a bit of a racking of the brain. It’s just that I learned the study before reading the normative”.	Stiff method: “If I have a difficulty and it says in the regulations that this is what I have to do, there’s nothing I can do! Nothing!”. Cost: “The company won’t want to expand money, employee time, and everything”. Study different from the regulations: “Sponsors won’t want to hire the study because it’s different from what the regulations say and that’s very difficult.”	Difficulty in finding people who work with the same method: "I don't know anyone who works with quails" "Acute oral toxicity".	Companies providing services	"Absolutely. It's very important. It's just that it's now come into force. But especially before, in these five years, wow, of course, they created Premasul, wow, without a doubt it was a great thing to disseminate, because there are laboratories that are still trying to disseminate this kind of thing. It's difficult. Because you look at the Guide, which isn't the case with me because I went in with the test already in place. But you look at the Guide and for me it's very simple. But for someone who doesn't work with it, where the lab hasn't implemented it yet, there are things that are very vague, there are things that you only learn in practice. There's a lot of that at BCOP.”	“When we go to do the BCOP, the lab stops. Not the whole lab, but we need a certain amount of mobilization because it’s a long test. It takes a considerable amount of time. So many studies that we could do at the same time, we can’t. Because of BCOP. But at the same time, we can ‘run’ several samples at the same time. That’s also a nice thing, “At least three people”.
Biologist 2	"You need to look up the validation protocols it mentions and really see the step-by-step process. There are methods that you can't do because it doesn't say the right amount of a reagent to use. They give you the 'bulk', but it seems that you standardize the times in your laboratory more or less when they don't."	-	Implementation of methods: "as much as Renama is doing with PremaSul, with the courses, not everyone can access them, I think this dissemination of implementing methods to have something more homogeneous, not everyone doing it one way".	"A lot of companies are looking for regulations. Or that have already been disapproved at some point, again with some requirement by Anvisa or that they want to put back on the market."	"Yes, we need to publicize what each laboratory is working on. I think that in Brazil, even more so in private companies, it's very 'in the box', nobody wants to divulge what they're doing, to share knowledge. But I think it's important to do a survey to find out what your partner is doing, what they've achieved or not. I think it would be possible to divide up research and the interests of companies and service providers".	-
Pharmacist 1	"I think that by reading the OECD, you can do it. I think you'll have to suffer a little at first to implement the technique. Until you get the hang of it".	The guide doesn’t give some important technical details: "but I think it falls a little short when it comes to detailing the practical side. So, we end up having to have other support materials, we end up having to resort to the validation report. The IIVS has an interactive channel where they show videos of these techniques and the BCOP video is very good, very cool. So, they teach us all the washing techniques, all the tricks we need to perform the technique. So, we end up having to resort to these parallel materials just to find out the details of the technique".	-	-	"Given the visibility of these methods today, both with the regulatory issue that Brazil has to adapt to by this year for the implementation of these methodologies and regulatory acceptance, I think Renama is the main body that can make this interface between the executor and the method, because we are in a scenario where we need to apply these methods and we don't have trained personnel to do this. And there are many methods, and each one has a different evaluation, a different outcome, a different endpoint. So, they're not at all similar methodologies. I think that this dialogue between Renama and researchers and the people who are going to carry out the techniques must take place. I think that the Premasul program itself is an attempt to reduce these gaps between these different means, but I think it's still too little, that, given the short time we have to adapt, I think actions need to be taken more often. I think Premasul's idea is very good, but I don't know if it will reach everyone it needs to reach in the time we have. But I think it's a start, a first step.”	"Short courses, such as a weekend covering all the technical details of ex vivo and in vitro methodologies, are essential. Each methodology has its own particularities, requiring technical mastery and, in some cases, specific structure and equipment, such as the opacitometer needed for BCOP. Promoting communication between laboratories and holding short courses can increase the reach and training of more professionals in less time, supplying specific market niches efficiently. Extensive courses, such as those offered by Premasul, are valuable, but due to their long duration and high cost, they end up limiting participation. Therefore, an approach focused on short, targeted courses can better meet the sector's emergency needs."

Continue

Continuation

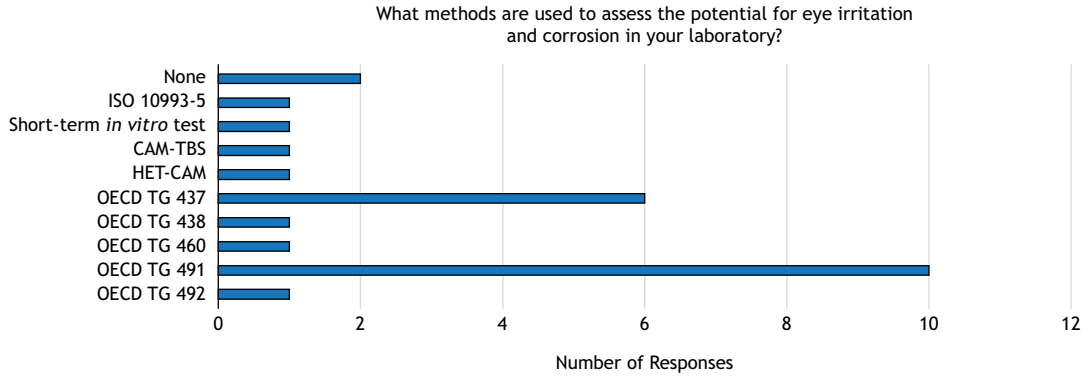
Veterinarian 1 and Biologist 3	"All guidelines are just a guide. If you want a more in-depth approach, you need to consult the literature cited at the end of the guidelines, where the validation has been published. I believe it should provide a clear enough procedure so that everyone can develop the technique without having to search for additional references. And sometimes, even then, we can't find what we need. You have to do a lot of research, take courses, training and look for other sources."	"If you want to have a more in-depth approach to the subject, you have to consult the literature at the end. This is where the validation was done and published. The guideline doesn't specify age for the animals. How can we guarantee this on a daily basis? Because as the eyes come from slaughterhouses, it's difficult to know the exact age of the animals. As it's in the guideline, we have to report it, but if we can't have this traceability. Another issue is the preparation of fluorescein, which needs to be in specific concentrations, but the diluent is unclear, whether it should be saline solution or another medium. These gaps in the guidelines result in difficulties in practical application, where adjustments are made based on daily mistakes and successes. In addition, there is a lack of detail about sample preparation, such as the inclusion of surfactants, which is not clearly described."	"Difficulty in finding people trained to do this, difficulty in finding inputs, there are a lot of things, let's say, the importation of tissues and in Brazil the view of importation is still seen as organ trafficking, so it's difficult to bring tissues into Brazil. I think that as long as Anvisa doesn't give its integrity to facilitate research or development, we're going to be stuck, stagnating in this part of technological advancement related to research."	-	V1: "Yes, it's very important even for our clients, because through it they can choose the laboratories that are accredited by Renama, if they take part in the inter-laboratory tests. Through these tests, the laboratory demonstrates its proficiency in carrying out the technique. So I think this kind of participation is very important."	“Eu acho que os cursos do Renama e Premasul são enxugados na parte prática. Eu acho que se fosse mais detalhado, procedimento por procedimento, acho que seria mais proveitoso. Uma coisa é você estar no dia a dia executando. Outra coisa é você estar vendo alguém fazer e de uma maneira mais encurtada fica difícil essa assimilação para depois quando você estiver no dia a dia executando a análise”.
Veterinarian 2	"You'll understand a good part of the regulations. I found it very difficult to understand the calculations when it came to the norms. From the moment we did the test today, putting the data into a table. When I applied the formulas, it became much clearer than the reading I did on the OECD".	About the guide being "stiffened": "I think they try to put an indication for each test, right. In the sense that you have a pre-established protocol. I think it's interesting because it gives you a guide to the use and functionality of a test. As soon as you delve deeper into the reading, the knowledge of the application of that test, you see that it has a wider range of functions or interpretations or bases that you can associate with other tests, to be put to better use than just in the OECD".	-	-	L: "I think it's extremely important for us to know about the existence of the methods, because often, even though I work in research, I used to work in veterinary science, it's still not a routine within some courses, even alternative methods, when you talk about alternative methods even within postgraduate programs, it's still a subject that isn't discussed as much, they're still not as widespread, the proper exploitation to try to make the maximum reduction in animals. It's important that the subject is more widely disseminated and that the more courses are offered, we can train ourselves to know that tests exist, what each test is for, and that we can bring this into our routine. I feel that there is a big difference between postgraduate programs. We see that there are programs within the same institution that have an extremely developed profile for alternative methods and some programs that should still be going through processes of reformulation, modernization and adaptation so that the application of these tests becomes more viable."	-

Continue

Continuation

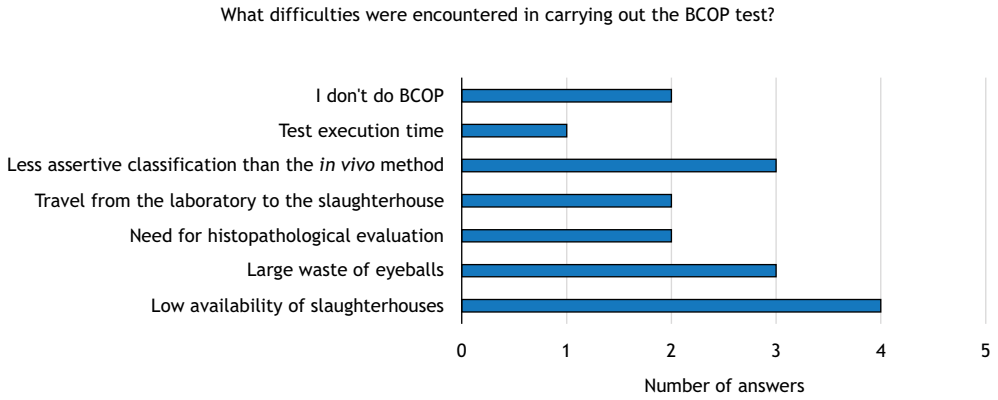
Veterinarian 3	"No. The Guide is not self-explanatory. The guide explains the basics of the procedure. But the details you either need to know beforehand or you need to know someone who already does the practice, or you need to look up the methodologies in scientific articles, but the guide is not self-explanatory."	"I think it's stiff, in the sense that my experience, citing a difficulty I've had basically in the dilution process. So, when I can't dilute my substance to what is indicated, I need to do a whole check on the diluent I use. And often in the Guide I don't have any other options. And I end up being a little afraid that it won't be accepted in the future. So even with the proof, because it turns out that I need to develop the methods of proof and I'm a bit afraid that it won't be accepted in the future, only with a scientific basis".	"The biggest obstacle has been the implementation according to the Guide with a small team and limited technical knowledge in cell culture. Other challenges will arise after overcoming this initial barrier. Alternative methods are new to everyone, and we are at a time of regulatory change. Implementing a validated method is difficult, with little possibility of modification. Here the difficulty increases with the import of equipment and reagents. We depend on local training, making the process more expensive and complex."	-	-	-
Pharmacist 2 biochemistry	"First, you need to study the protocol, the subject, and the validation document, which is usually in the protocol references. The validation document for the technique provides much more information and is more detailed. Implementing a test in a laboratory is not an easy task; it requires a lot of study of the techniques, the possible biases, the field of application of the technique, the substances, and requires expertise in the field. We have implemented various protocols, but the existence of training makes it much easier."	x	"Alternative methods are new to everyone. We're going through a regulatory change, a paradigm shift, so everything is very new. And implementing a method on its own is difficult in order to meet regulatory criteria, because you have to meet all the criteria of the method, and it's a formatted method, it's a method that has been validated. So, you don't have much possibility of modifying this method. Specifically in Brazil, the difficulty is even greater because we need both imported equipment and reagents and the cost of this method for us is much higher compared to other countries. So, as well as depending on training in Brazil, we have to import these methods and they cost us more."	Research and a bit of regulation.	"I think Renama is very important because it brings together people with common interests. And by bringing together people with common interests, we can organize and help each other in this implementation, clearing up doubts, passing on processes, training people. So, I think Renama is very important as a promoter of this".	"There are several bottlenecks that we still need to try to develop methodologies. In general, I think we have little incentive, because trying to develop a methodology is very expensive, and even when you develop a methodology, in the case of a method we developed for corneal tissue, we would need much more investment to carry out inter-laboratory validation, and then this could meet regulatory criteria in Brazil. So, we'd need a lot more investment, because we're developing a methodology and then validating it. This is a long and expensive process. So, we don't have that kind of investment earmarked for this. And we don't have easy access to reagents either. We buy imported antibodies, and everything is much more expensive for us, as well as equipment. Another thing is the legal side. We have some legal obstacles, the very use of tissues, so all of this has to be reviewed in Brazil. The legal framework, the conditions and acquisition of reagents, and more investment in the development of methodologies in Brazil."

Source: Prepared by the authors, 2023.



Source: Prepared by the authors, 2023.

Figure 1. Percentage of responses to the question "What methods are used to assess the potential for eye irritation and corrosion in your laboratory?" (N = 17 respondents).



Source: Prepared by the authors, 2023.

Figure 2. Percentage of responses to the question "What difficulties were encountered in carrying out the BCOP test?" (N = 8 respondents).

(13 respondents) with a focus on the practical side (nine respondents or 40.9%). The difficulty of finding people who work with the same method to network and exchange information was pointed out by approximately 36% of respondents (eight) (Figure 3).

An open question was also asked: "Could you suggest a way to improve the tests carried out by your laboratory?", with a total of 10 respondents. The answers included: (i) the need for investment (funds) to purchase quality materials, equipment, and consumables (60%); (ii) investment in human resources, such as training, capacity building, and more civil servants (50%), followed by (iii) the need for greater contact with Renama and regulatory agencies (10%), (iv) the difficulty of importing materials (10%), and (v) the difficulty of implementing good laboratory practices (10%).

Interviews

The second stage of the study involved seven participants. The transcripts of the interviews are summarized in the table in Chart 2.

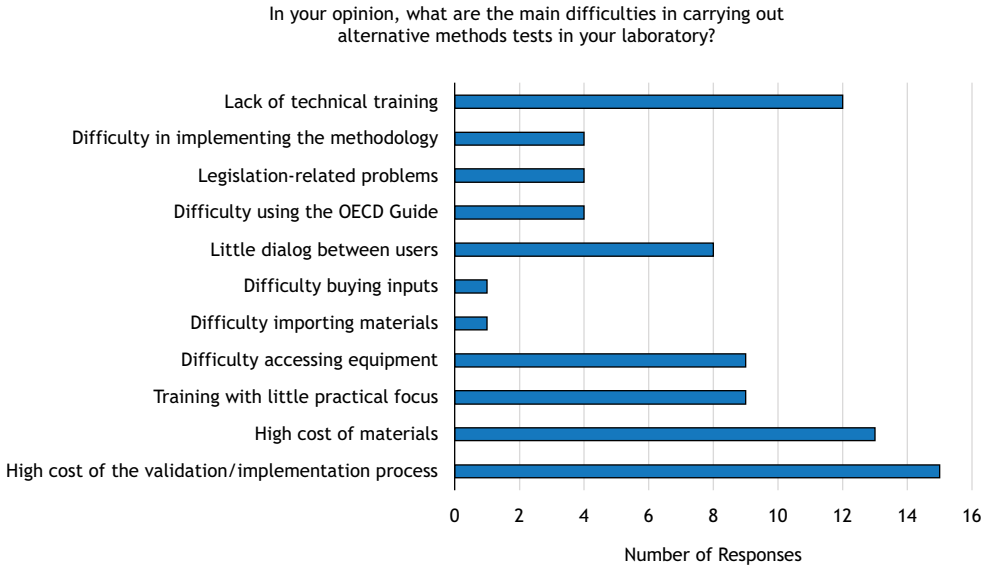
The main difficulties faced by the executors of the BCOP methods were related to the following factors.

Little availability of eyeballs from the slaughterhouse

Biologist 1 said that with NR No. 18 (Concea), the demand for BCOP increased and the slaughterhouses "didn't have any availability because they were already going to sell to another laboratory. We just needed contacts of slaughterhouses that could meet our demand". **Biologist 2** said that "we didn't always get the eyes. When we were going to collect, we had to give plenty of notice".

Veterinarian 1 and **Biologist 3** reported that the difficulty of supply was related to the need to incinerate the material: "We used to have two local suppliers, but they argue that the eye is a source of contamination. So, they can't pass on the bulls' eyes to our laboratory because they need to guarantee that the eyes will be incinerated in the end."

This same difficulty was presented by **Biologist 1,** when they said that "the meatpackers aren't even willing to sell this eyeball to us because they're worried about fungal encephalopathy, which is mad cow disease" and "[...] It has to be incinerated. And as much as we tell the slaughterhouse that we can get a certificate, a letter from the laboratory saying that it's for research purposes, they still won't accept it. So, it's been a bit complicated.



Source: Prepared by the authors, 2023.

Figure 3. Percentage of responses to the question "In your opinion, what are the main difficulties in carrying out alternative methods tests in your laboratory?" (N = 22 respondents).

Eyeball waste

The great waste of eyeballs was another factor discussed. Of the four different laboratories from which the respondents come, three buy eyeballs and one receives them as a donation. The average amount paid per laboratory was R$ 3.00 per globe in 2020. The average number of eyeballs requested per visit by users was 43 ± 13.

They all said there was a lot of waste of eyeballs caused by scratches, opacity, and other damage. **Veterinarian 1** and **Biologist 3** said that "as the oxen's eyes are removed in the fridge, they usually come with scratches, or with a very small diameter that you can't get the right diameter to fit inside the holders. That's why we always order extra so that we don't run out on the day".

Biologist 1 states that

> "When we make the request, it's usually around 40 eyeballs. They arrive at the laboratory, and when we go to do a macroscopic assessment there are many corneas that are damaged, have a scratch, something that prevents us from using them in the test. So, I think this difficulty happens more because the collection is done by an employee of the slaughterhouse. So, it doesn't have the same effect as if it were the technician doing the collection. So, we 'waste' a lot of corneas. I end up using about 18."

Difficulty in classifying the method

Another topic addressed was the fact that the BCOP method does not provide such an assertive classification with this method. **Biologist 1** stated that they "can't get as assertive a classification as *in vivo*". This would be a problem because this method evaluates irritants and non-categorized (non-irritants). As a solution to this problem, **Pharmacist 1** suggested that "histopathological evaluation should be considered for inclusion in the Guide, because I think it is the bottleneck that can help separate the categories more".

Travel from the laboratory to the slaughterhouse

Another point to highlight was the travel from the laboratory to the slaughterhouse. Half of the interviewees said that distance was not a problem, with the slaughterhouse located within an average radius of 78.3 km, involving an average travel time of 1 h 30 min from the laboratory to the slaughterhouse. However, **Biologist 1** said that "we start the test the next day, because there's no time".

Technical execution problems

Technical problems in carrying out the test were also pointed out in the interviews. We categorized technical problems as issues related to the time spent washing the material during the test, which takes a long time to perform manually, the time and difficulty of washing with colored products, the fact that the material could not be removed with just the washes recommended by the Guide, and the time it took to perform the methodology itself. This is clear from **Biologist 1**'s account:

> "When we started doing the technique, we didn't have it automated, with the suction pump. So, we did everything by syringe and that took up a lot of time. And since we worked with a lot of samples, we took on a project in which we had 86 pesticide samples to evaluate, this was a problem because there were a lot of washes and so on. When we bought the pump, which isn't that expensive and has almost no maintenance, it saved us about 2 hours of testing in the same day."

> "Many pesticides we analyzed were colored. They were blue, red, so sometimes with just the number of washes described in the Guide we couldn't completely remove the substances. So, we had to do several washes and sometimes they remained impregnated on the surface of the cornea. Because some of these substances had ingredients that I don't even know if they were organic molecules. It seems that there are some inorganic things that impregnate there and it's very difficult to get them off. So, I think this is a technical limitation."

Another difficulty with the test is the time it takes to run the samples. **Biologist 2** says that "depending on whether it's a liquid or a solid, it takes longer, and you spend more time in the lab, because you have to finish the readings that day". **Biologist 1** says that because the test is so long, other employees have to stop their work to help them carry out the method:

> "When we go to do the BCOP, the lab stops. Not the whole lab, but we need a certain amount of mobilization because it's a long test. It takes a considerable amount of time. So many studies that we could do at the same time, we can't. Because of BCOP. But at the same time, we can 'run' several samples at the same time. That's also a nice thing, but we always have at least three people."

Difficulties in implementing the methodology

The difficulties of implementing the methodology were addressed by **Veterinarian 3**: "I think that currently my biggest obstacle has been the implementation of trying to do it according to the Guide. As my team is very small and we have somewhat limited technical knowledge in the area of cell culture, in the more specific area, I've had a bit of difficulty extrapolating."

Pharmacist 1 addressed the issues surrounding the implementation of the methods due to regulatory acceptance for the replacement of alternative methods and the need for more training:

> "Given the visibility of these methods today, both with the regulatory issue that Brazil has to adapt to by this year for the implementation of these methodologies and regulatory acceptance, I think Renama is the main body that can make this interface between the executor and the method, because we are in a scenario where we need to apply these methods and we don't have trained personnel to do this. And there are many methods, and each one has a different evaluation, a different outcome, a different endpoint. So, they're not at all similar methodologies. I think that this dialogue between Renama and researchers and the people who are going to carry out the techniques must take place. I think that the Premasul program itself is an attempt to reduce these gaps between these different means, but I think it's still too little, that, given the short time we have to adapt, I think actions need to be taken more often. I think Premasul's idea is very good, but I don't know if it will reach everyone it needs to reach in the time we have. But I think it's a start, a first step."

Pharmacist 2 adds on the issue of regulatory change and implementation of the method:

> "Alternative methods are new to everyone. We're going through a regulatory change, a paradigm shift, so everything is very new. And implementing a method on its own is difficult in order to meet regulatory criteria, because you have to meet all the criteria of the method, and it's a formatted method, it's a method that has been validated. So, you don't have much possibility of modifying this method. Specifically in Brazil, the difficulty is even greater because we need both imported equipment and reagents and the cost of this method for us is much higher compared to other countries. So, as well as depending on training in Brazil, we have to import these methods and they cost us more."

Using the OECD Guide

Regarding using the OECD Guide, all the interviewees mentioned some kind of difficulty. Various obstacles were mentioned in relation to the stiffness of the method, including the impossibility of making changes to the protocol, as some of them work for private companies and are afraid of changing the protocol and the requester not accepting this modification; lack of technical detail on the practical part, the need to look for other support materials, such as the method validation material.

According to **Biologist 1,** "If I have a difficulty and it says in the regulations that this is what I have to do, there's nothing I can do! Nothing!"; "the company won't want to expand money, employee time, and everything"; "sponsors won't want to hire the study because it's different from what the regulations say and that's very difficult".

Pharmacist 1 points out:

> "The guide doesn't give some important technical details, but I think it falls a little short when it comes to detailing the practical side. So, we end up having to have other support materials, we end up having to resort to the validation report. The IIVS has an interactive channel where they show videos of these techniques and the BCOP video is very good, very cool. So, they teach us all the washing techniques, all the tricks we need to perform the technique. So, we end up having to resort to these parallel materials just to find out the details of the technique."

For the **Biologist 3:**

> "If you want to have a more in-depth approach to the subject, you have to consult the literature at the end. This is where the validation was done and published. The guideline doesn't specify age for the animals. How can we guarantee this on a daily basis? Because as the eyes come from slaughterhouses, it's difficult to know the exact age of the animals. As it's in the guideline, we have to report it, but if we can't have this traceability, it's difficult. Another issue I'd like to mention that the guideline doesn't mention is how Fluorescein is prepared. It says that it must be prepared at a

> concentration of 4 mg/mL for liquids and 5 mg/mL for solids. But what is the diluent? Is it the physiological solution? Is it the medium itself? So, this is poor information. It's the day-to-day work that makes you realize your mistakes and successes and improve your technique."

Veterinarian 1 adds: "It's not exactly described in terms of the samples. Whether there should be surfactant or not, how do we prepare this sample for application". **Veterinarian 3** states that:

> "I think it's stiff, in the sense that my experience, citing a difficulty I've had basically in the dilution process. So, when I can't dilute my substance to what is indicated, I need to do a whole check on the diluent I use. And often in the Guide I don't have any other options. And I end up being a little afraid that it won't be accepted in the future. So even with the proof, because it turns out that I need to develop the methods of proof and I'm a bit afraid that it won't be accepted in the future, only with a scientific basis."

Cost, investment, and legislation

We can see, especially among the professionals interviewed from public laboratories, the need for investment and the high cost of imported materials, as well as the high cost of a validation process, as difficulties raised. According to **Pharmacist 2:**

> "There are several bottlenecks that we still need to try to develop methodologies. In general, I think we have little incentive, because trying to develop a methodology is very expensive, and even when you develop a methodology, in the case of a method we developed for corneal tissue, we would need much more investment to carry out inter-laboratory validation, and then this could meet regulatory criteria in Brazil. So, we'd need a lot more investment, because we're developing a methodology and then validating it. This is a long and expensive process. So, we don't have that kind of investment earmarked for this. And we don't have easy access to reagents either. We buy imported antibodies, and everything is much more expensive for us, as well as equipment. Another thing is the legal side. We have some legal obstacles, the very use of tissues, so all of this has to be reviewed in Brazil. The legal framework, the conditions and acquisition of reagents, and more investment in the development of methodologies in Brazil."

Similarly, for **Biologist 3:**

> "There are a lot of things, let's say, the importation of tissues and in Brazil the view of importation is still seen as organ trafficking, so it's difficult to bring tissues into Brazil. I think that as long as Anvisa doesn't give its integrity to facilitate research or development, we're going to be stuck, stagnating in this part of technological advancement related to research."

Need and access to more courses and training

The need for and access to more courses and training in the methodology (capacity building) and implementation of the method were highlighted by the interviewees. The difficulty in finding qualified people was addressed by **Veterinarian 1** and **Biologist 3**, and **Biologist 2** said "as much as Renama is doing with PremaSul, with the courses, not everyone can access them". Regarding training and implementation, **Veterinarian 1** and **Biologist 3** stated that

> "The Renama and Premasul courses are short on practical aspects. I think that if they were more detailed, procedure by procedure, it would be more useful. It's one thing for you to be doing it on a day-to-day basis. It's another thing if you're watching someone else do it, and in a shorter way it's difficult to assimilate it when you're carrying out the analysis on a day-to-day basis."

According to **Biologist 2**, "As much as Renama is doing with PremaSul, with the courses, not everyone can access them, I think this dissemination of implementing methods to have something more homogeneous, not everyone doing it one way".

Dissemination of alternative methods in undergraduate and postgraduate courses

The issue of disseminating alternative methods in undergraduate and postgraduate courses was also identified, as **Veterinarian 2** pointed out:

> "I think it's extremely important for us to know about the existence of the methods first, because... when you talk about alternative methods, even within postgraduate programs, it's still a subject that isn't discussed as much, they're still not working as much, they aren't properly explored so that we can try to reduce the number of animals as much as possible. It's important that the subject is more widely disseminated and the more courses that are offered... I feel there's a big difference between one postgraduate program to another. We see that there are programs within the same institution that have an extremely developed profile for alternative methods and some programs that should still be going through processes of reformulation, modernization and adaptation so that the application of these tests becomes more viable."

This comment highlights the importance of making people aware of the existence of alternative methods in research, particularly in postgraduate programs, to promote greater dissemination and discussion on the subject.

DISCUSSION

Historically, animal tests have been used to determine the level of ocular toxicity as standard practice for decades[6]. However, due to ethical and legal concerns and advances in biotechnology, there has been an increase in the development of alternative methods that can predict the toxicity of chemicals with reduced or no use of animals[27]. While there are challenges associated with implementing alternative methods, there are also numerous associated opportunities. Understanding this scenario strengthens initiatives and supports the creation of new knowledge.

To this end, one of the most valuable strategies for gathering in-depth information is the use of qualitative research such as interviews[40,43]. Based on the data from this study, it was possible to carry out a diagnosis with alternative methods practitioners in Brazil, identifying some of the main challenges, difficulties, and limitations. In addition, secondary themes were identified that could open opportunities for scientists to undertake innovative problem-solving[44], such as applying courses or offering services, with biotech deep techs accounting for 61% of the sector in Latin America and the Caribbean[45].

Research with limited resources is a task that requires initiative and creativity to adapt knowledge to obstacles such as lack of materials, investment and lack of incentives. This is a worldwide problem, and certainly of great relevance in Brazil[46]. Some of the limitations for animal substitution are: the lack of specific legislation on the use of biological material of human origin for toxicological tests, making it difficult to access alternative *in vitro* models[47]; difficulties related to the long process of accepting an internationally validated methodology into Brazilian legislation; the long process of validating an alternative method, which can take an average of 10 to 15 years[46].

Another limitation is the fact that a single *in vitro* method cannot replace *in vivo* testing and predict all toxic categories of chemicals. However, a strategic combination of several alternative methods within a testing strategy may be able to replace animals[30]. In cases of difficult classification for ocular or cutaneous toxicological evaluation, for example, histopathological evaluation is used[48]. As a challenge faced by this same outcome, we can cite the issue of slaughter centers, as they are usually far from the method executors, making it difficult to get the material to the laboratory, as well as the lack of public funding for Brazilian research and method development.

An important aspect identified in Biologist 1's account, when they say that "we start the test the next day, because there's no time", indicates that geographical distance is a factor that makes it difficult to carry out experimental tests within 24 hours of collecting samples. Studies point to the use of unmanned aerial vehicles (UAVs) as an innovative solution to the problem of transporting biological samples, which have been tested and evaluated in different scenarios around the world[50,51]. The Oswaldo Cruz Foundation (Fiocruz) has pioneered work in this area by proposing a logistics model using UAVs for Public Health[51]. In addition, the literature lacks experimental studies validating the extended use of eyeballs after 24 hours of collection, allowing eyeballs to be used more sustainably and for longer, and it is also necessary to identify suitable preservation protocols for this test.

It is also worth noting that slaughterhouses produce a large amount of biological waste and, most of the time, a large part of this material is underutilized and disposed of incorrectly, such as incineration or disposal in dumps or landfills[53,54]. Previous studies have highlighted various beneficial applications for waste from slaughterhouses[54,55,56], with animal recycling being an indispensable activity for the sustainability of the animal protein production chain. Collecting and correctly disposing of the waste produces products for other industries, such as animal feed, agriculture, the chemical and petrochemical industry, and the hygiene and beauty industry[57]. Although the literature shows that there is a large supply of this material, our research showed that some researchers had difficulties in obtaining biological material because there are not many slaughterhouses in the region, as well as a concern and resistance on the part of slaughterhouses to donate this material for fear of zoonoses.

Another relevant issue pointed out in this study was the large amount of eyeballs wasted due to scratches, reduced diameter and damage caused when the material was collected after being received in the laboratory and previously evaluated for the BCOP test. As a solution to the problem of waste with a view to sustainability, Khan et al.[58] suggest the development of a collaborative institutional training initiative (CITI), in which trained staff or volunteers must be present at the time of removal. The CITI modules allow workers to be guided precisely so that organ extraction is more successful. In this initiative, before the extraction, researchers would learn the procedures for handling and transporting tissues through mandatory training. All procedures would be supervised by the lead researcher and researchers would be instructed to follow the abattoir's policies, at the risk of being excluded from the sample collection process.[58]

Some reports from participants in this study point out that the BCOP method does not provide as assertive a classification as the *in vivo* method. On the other hand, the assessment of the depth of damage in isolated corneas was proposed by Maurer et al.[59] to predict the degree and duration of tissue damage[58]. The additional characterization of the damage by histopathological evaluation also helps to identify cases in which the response is on the borderline between two categories based on the decision criteria of the method[59,60]. Accordingly, OECD Guidance No. 160 recommends that users preserve tissues for histopathological evaluation in order to follow the depth of injury for a better understanding of eye damage, using the hematoxylin and eosin (H&E) method[48], Jeong et al.[61] noted that traditional H&E staining may not provide sufficient information to classify eye-irritating chemicals. Thinking of an improvement, the authors carried out the BCOP test with chemicals with known results and proposed the histopathological evaluation of the corneal structure using three staining methods H&E, Masson's trichrome, and periodic acid schiff[61]. Therefore, the histopathological evaluation of corneas appears to be an important complementary technique to the BCOP to provide the necessary information for a more assertive classification, such as that sought by the survey respondents.

Several obstacles have been mentioned in relation to the use of the OECD Guidelines. In general, the OECD guidelines for alternative methods offer a set of robust principles and criteria for validating and assessing the reliability and relevance of non-animal tests for regulatory purposes. However, the present reports point to a perception of inflexibility in the proposed procedures, due to the impossibility of changing the protocol because some users

provide services for private companies and are afraid that the modification of the protocol will not be accepted by the client, due to the risk of regulatory non-acceptance. In addition, the execution time factor during the test was pointed out critically in the survey, especially regarding the number of washes and the removal of colored substances, which are not recommended in the OECD 437 Guide. In addition, the lack of detailed technical information during practical execution was highlighted, requiring users to seek other support resources, including the original method validation documents. Although all the interviewees pointed out some difficulty in using the OECD Guide for BCOP, there are no similar reports in the scientific literature. This topic is of great importance and requires more in-depth analysis to contribute to the wider dissemination and use of these methods.

The open question "Could you suggest a way to improve the tests carried out by your laboratory?" allowed us to directly identify opportunities for improvement in this application. These include the need to implement good laboratory practices and the importance of attracting and training a permanent technical team, reducing dependence on temporary staff such as scholarship students. To this end, it is essential to provide adequate training in the use of alternative methods.

Although Brazil leads PReMASUL, which was created in 2015 by the MCTI with the aim of offering courses to disseminate the concept of "alternative methods" in Mercosur countries, promote the development of laboratory infrastructure and train specialized professionals, our survey revealed opportunities for improvement. Among the respondents in the area, 59.1% expressed the need for more courses and practical training in methodologies and the implementation of alternative methods. In addition, 36.4% highlighted the importance of establishing connections with other professionals who work with the same methods to network and exchange information. Another important aspect to consider was the need to disseminate alternative methods in undergraduate and postgraduate courses. In addition to promoting the creation of PReMASUL, Brazil was a pioneer in establishing *lato sensu* postgraduate courses in alternative methods, with the country's first specialization course in the area offered since 2019 by Fiocruz's Institute of Science and Technology in Biomodels (ICTB).The program was created with the premise of offering consolidated basic training, focused on the concepts of bioethics, animal experimentation, alternative methods and validation, in a related and applied way[27,62]. Such initiatives also stand out as an opportunity for scientific entrepreneurship.[44]

This survey also identified various challenges related to costs and financial resources. It can be seen that 68.0% of the interviewees mentioned the high cost associated with the validation process, followed by 59.1% who pointed out the high cost of materials and 40.9% who highlighted the challenges related to acquiring equipment. To address these issues, it is important to: seek ways to make the import and purchase of inputs more accessible, improve laboratory infrastructure, invest in more robust equipment and establish effective maintenance and calibration practices. In addition, promoting the development of public policies to guarantee adequate funding and strengthening partnerships with entities such as Renama and regulatory agencies, as well as encouraging an organized and protected input production chain can represent a promising path to improving this situation.

This study revealed a series of challenges and opportunities related to the use of alternative methods in ocular toxicology research. Despite all the difficulties pointed out, it is important to note that this research offers valuable contributions to the literature by documenting the practical experiences, the challenges faced by users of alternative methods in Brazil and the opportunities that can be opened to mitigate the difficulties encountered by researchers. These findings can serve as a basis for future research and the formulation of public policies aimed at improving the effectiveness and adoption of these methods, promoting more ethical, efficient and sustainable research in the country, strengthening the ecosystem of open innovation and digital transformation based on the Legal Framework for Innovation, with a fundamental role for the adoption of such practices in the local policy of its technological innovation hubs[63].

One of the main limitations of the study concerns the sample size. Although the sampling procedure was carefully planned to include a wide variety of experiences, our sampling procedure was limited to the list of official laboratories registered with Renama and to participants approached at events on the subject. However, our conclusions can only be generalized to this study group, and the pattern of responses may differ in other countries or if we were able to approach a larger universe of respondents. Although rigorous data collection and analysis methods were employed, such as semi-structured interviews and thematic analysis, it is possible that other methods could have provided a more in-depth or complementary understanding of the participants' experiences and perceptions. Future research could benefit from broader samples, including laboratories not yet registered with Renama and from other countries, with research support from the Alternative Methods Validation Centers in each country, with the same methodological standard of data collection and analysis, to obtain a more comprehensive and accurate understanding of this topic.

We hope that mapping the institutions that carry out alternative methods in Brazil will help to ensure that these methods are implemented and replaced, strengthening initiatives on the importance of participating in collaborative networks such as Renama and BraCVAM (Brazilian Center for the Validation of Alternative Methods). PReMASUL also plays an important role in placing Brazil and other Mercosur countries on the path to research and development of alternative methodologies. These initiatives enable partnerships with national and international laboratories for multicenter research, training in techniques of interest, and a positive impact on public health research demands. In addition, notices for innovation support, such as Inova Fiocruz, can play a crucial role in strengthening and implementing alternative methods to the use of animals in research and development, offering funding and essential resources so

that research institutions and laboratories can overcome some of the main challenges identified in implementing these methods.

Previous studies have highlighted other valid methods not yet published in international guidelines, such as those of the OECD, but which are being used to assess ocular effects, pointing to the need to validate models and further develop methods for applications in ocular diseases and to provide information on the reversibility of effects[24,64,65,66]. Regarding BCOP, several of the challenges encountered in our study connect with those that have been reported in reviews of international guidelines for performing BCOP. These include high false positive rates for some chemical groups and categories of eye irritants, requiring additional tests for definitive classification, detection of reversibility, and systemic toxicity associated with eye exposure. Recent revisions of the International Guide also point to the need to minimize the interval between the collection and use of corneas in the BCOP, usually performing both on the same day. Therefore, in an international context, it is likely that BCOP users in other countries experience some conditions similar to those described here, a hypothesis to be confirmed only in future studies.

CONCLUSIONS

The survey revealed a series of challenges faced by Brazilian laboratories in implementing alternative methods for assessing eye irritation, especially BCOP. The main difficulties include: the high costs of validating and importing materials, the need for more training, traveling to slaughterhouses, and the limited availability of eyeballs. Despite these barriers, the adoption of alternative methods is essential to promote more ethical and sustainable practices in toxicological research. The creation of public policies that facilitate the import of inputs, the strengthening of partnerships between research institutions, and the provision of adequate funding are fundamental steps to overcome these difficulties. In addition, the dissemination of these methods in undergraduate and postgraduate courses can promote greater awareness and adoption of these practices. Future actions in the implementation of the BCOP can focus on the interactions of important institutional players in the production of technical adaptations, the production of documents, and revisions of current guides, and studies that can expand its usefulness as an alternative method in the assessment of ocular toxicity.

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Acknowledgements

The authors gratefully acknowledge the financial support of Inova Fiocruz, Rio de Janeiro, Brazil, and the National Research Council (CNPq-Brazil), of which Gutemberg Gomes Alves is a research productivity grant holder 2.

Authors' Contribution

Presgrave OAF, Gonzalez MS, Alves GG - Conception, planning (study design). Gimenes I - Acquisition, analysis, data interpretation, and writing of the paper. All the authors approved the final version of the paper.

Conflict of Interest

The authors inform that there is no potential conflict of interest with peers and institutions, political or financial, in this study.