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A importância do controle de qualidade de culturas utilizadas em ensaios biológicos e no desenvolvimento de pesquisas na área de saúde

The importance of quality control of cultures used in biological assays and in development of research in the health area

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ABSTRACT

Introduction: Advances in scientific research are based on previously published findings. However, there is concern about the lack of reproducibility in the biological researches in basic and pre-clinical areas, due to the repercussion on the population´s health. Because in vitro cultured cells are the basis for many toxicological and therapeutic studies, concern about their quality becomes paramount. Regarding microbiological contaminants, although bacteria and fungi are easily recognized, viruses and mycoplasmas are invisible under light microscopy. Another delicate issue would be the results generated with cells with modified identity. **Objective:** To discuss the main methodologies for assuring the quality of cells used in in vitro assays and to demonstrate how some world collections are structured to address this issue. **Method:** The scientific literature in the PubMed and Scielo databases and the webpage of different biological collections until December 2017. **Results:** It is recommended to apply the following techniques to detect contaminants in cell cultures: 1) virus : PCR and viral isolation; 2) mycoplasmas: PCR, bioluminescence and staining of cells with DNA affinity fluorophore; 3) human cell identity: the STR; 4) non-human cell identity: the Barcode. **Conclusions:** Considering all the investment applied in scientific research worldwide, the development of new methodologies alternatives to the use of animals and the critical consensus of the concept of quality, it is concluded that any laboratory should guarantee the control of purity and authenticity of its lineages.

KEYWORDS: Cell Lines; Purity; Authenticity; Reproducibility

RESUMO

Introdução: Avanços na pesquisa científica baseiam-se nas descobertas previamente publicadas. Entretanto, há preocupação com a falta de reprodutibilidade nas pesquisas biológicas das áreas básica e pré-clínica, em função da repercussão na saúde da população. Como células cultivadas in vitro constituem a base para muitos estudos toxicológicos e terapêuticos, a preocupação com a qualidade destas torna-se primordial. Com relação aos contaminantes microbiológicos, embora bactérias e fungos sejam facilmente reconhecidos, vírus e micoplasmas são invisíveis na microscopia óptica. Outro problema delicado seriam os resultados gerados com células com identidade modificada. **Objetivo:** Discutir as principais metodologias para a garantia da qualidade de células utilizadas em ensaios in vitro e demonstrar como algumas coleções mundiais estão estruturadas para tratar esta questão. **Método:** Levantamento da literatura científica nas bases de dados PubMed e Scielo e na página da web de diferentes coleções biológicas até dezembro de 2017. **Resultados:** Recomenda-se a aplicação das seguintes técnicas para detecção de contaminantes em cultivos celulares: 1) vírus: o PCR e o isolamento viral; 2) micoplasmas: o PCR, a bioluminescência e a coloração das células com fluoróforo com afinidade ao DNA; 3) identidade de células humanas: o STR; 4) identidade de células não humanas: o Barcode. **Conclusões:** Considerando todo o investimento aplicado em pesquisa científica em âmbito mundial, o desenvolvimento de novas metodologias alternativas ao uso de animais e o consenso crítico do conceito de qualidade, conclui-se que qualquer laboratório deve garantir o controle de pureza e autenticidade de suas linhagens.

PALAVRAS-CHAVE: Linhagens Celulares; Pureza; Autenticidade; Reprodutibilidade

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INTRODUCTION

Advances in research are based on the reproducibility of data and on previously published findings. For this reason, there is a growing concern with the lack of reproducibility in research in general, especially in basic biological and pre-clinical areas because of its impact on public health. In life sciences, one of the most important contributors to the lack of reproducibility is the use of misidentified cell lineages isolated from different human sources or contaminated by microorganisms like mycoplasmas (intra and interspecies cross contamination)[1,2]. The lack of reproducibility affects an important aspect of scientific experimentation, because if experiments cannot be reproduced, they are useless at all, eventually implying in waste of time and money.

The cultivation of cells has been developing exponentially since the 19th century to the point that, today, cells grown *in vitro* serve as therapeutic tools[3,4,5], models for the study of various phenomena and biological processes[6,7,8,9] and toxicological studies[10,11,12,13,14], among other applications. Still in the context of cell cultivation and its applications, the contribution of the *in vitro* studies in regulatory science for the assessment and registration of products is undeniable. These studies must fulfill high quality standards in order to obtain reliable results with acceptable reproducibility.

A recent survey done by Nature[15] revealed contradictory attitudes of researchers towards the lack of reproducibility because, although more than half of the scientists agree with this problem, only about 30% recognize that this is due to wrong data. In fact, many consider that the most important factors for the lack of reproducibility are the competition and pressure to publish, followed by poor statistical analysis and experimental design, among other factors. Only a third of the respondents reported strategies to improve the reproducibility (repeating the study or asking someone else to do it).

For example, in the field of cancer biology, the initiative called Reproducibility Project: Cancer Biology (https://elifesciences.org/collections/9b1e83d1/reproducibility-project-cancer-biology - verified on 10/24/2017), led by the Center for Open Science (http://centerforopenscience.org/ - verified on 10/24/2017) and Science Exchange (https://www.scienceexchange.com/ - verified on 10/24/2017), aims to independently replicate selected results from several high level articles on cancer biology. Before data collection of the study to be replicated, the experimental design and protocols are peers reviewed and published; then, the results are published as *Replication Study*.

Even though this strategy is interesting, one can expect to first prevent flaws in the *in vitro* studies by paying attention to two important factors: the authenticity of the cells used in the study and the absence of microbiological contamination. In other words, the implementation of routines to control the quality of cell lineages used in scientific research is needed[16]. Recently, a study showed that the most common causes of laboratory errors that led to retractions are contamination (microbiological and incorrect identification) and analytical errors[17]. Thus, the objective of this review is to present the state of the art regarding the impact of microbiological contaminants and the authenticity of cells in health research.

METHODS

We conducted this study as an integrative review according to a previously described methodology[18], by surveying scientific articles between July 3 and October 30 of 2017, aiming to address the main aspects of cell quality and its impact on the reproducibility of scientific research. To elaborate this article, we followed these steps: 1) presenting the reader to the main microbiological contaminants in mammalian cell cultures; 2) discussing the importance of the authentication of cell lineages identity in laboratory tests; 3) verifying on web pages of various biological collections of the world how the information of quality control of the commercial biological material is available. We surveyed the scientific literature by consulting PubMed and SciELO databases, using keywords like: cell culture techniques/methods, quality control, reproducibility of results, cell authenticity, mycoplasma, cellular contamination. We included articles published in English and Portuguese without restriction of year of publication.

RESULTS AND DISCUSSION

Microbiological contaminants in mammalian cell cultures

The microbiological contaminants most commonly found in cells are mycoplasmas, viruses, bacteria and yeasts. In most cases, contamination by bacteria and fungi is easily recognized. If it is not possible to dispose of these cultures, they can be treated with antibiotics. However, contamination with mycoplasmas and viruses often goes unnoticed because they are invisible in direct observation under light microscope and not always cause changes in the morphology of the cells. Even so, reduction of growth rate, chromosomal abnormalities or changes in the metabolism of amino acids and nucleic acids can occur. For these reasons, contamination of cell cultures may call into question the results of *in vitro* trials. It may cause delays and financial losses and require efforts to detect and eliminate them when possible[19].

In the case of contamination by mycoplasmas, treatment of cell cultures is possible using one or more associated antibiotics in in-house protocols or even commercial products for this specific purpose. However, it is possible that the cells selected at the end of the treatment show some differences from the original culture[20]. Thus, we recommend that, after decontamination, the culture performances are evaluated as to the specific tests for which they will be used. For this reason, when possible, we recommend the preference for the disposal of the contaminated culture and replacement by a batch previously tested, free of mycoplasmas. In cases of viral contamination,

this disposal is the only option, since there is no effective way of decontamination.

In addition to the cells that originated the cultures (cell lineages or primary cultures), other animal ingredients used to maintain cell cultures, like fetal bovine serum (FBS) and trypsin, may be the source of viruses or mycoplasmas, as well as inadequate handling procedures. Due to its consequences or to the difficulty (or even impossibility) of decontamination, the prevention of microbiological contamination is the best solution to this problem. For this, the periodic monitoring of possible contaminants in cell cultures and their inputs is essential to maintain the quality of cell cultures and guarantee its reliance in *in vitro* test results.

In Brazil, Oliveira *et al.* developed a methodology for detection of some adventitious agents like mycoplasma, porcine circovirus 1 (PCV1), bovine viral diarrhea virus (BVDV)[19] and porcine parvovirus (PPV)[21] through polymerase chain reaction (PCR) and surveyed these contaminants in 88 cell cultures, 13 samples of FBS and ten samples of trypsin used in eight Brazilian veterinary laboratories (for routine testing or research). The results showed the following cell contamination rates: 34.1% with mycoplasma, 35.2% with PCV1, 23.9%, with BVDV and more than 50% with PPV. The genome of BVDV was detected in two samples of FBS and PCV1 in one sample of trypsin. These results demonstrated that the cell cultures, serum and trypsin used by different laboratories have a high rate of contaminants. It is worth highlighting that mycoplasma DNA was detected in cells of all laboratories, BVDV RNA in half of them and only 4.5% of the analyzed cell cultures were negative for all of the contaminants assessed. We attribute the results to the common practice of exchanging cell samples between laboratories and to the use of FBS or trypsin without prior assessment of contaminants. We demonstrate the need for control of biological contaminants in laboratories and cell banks.

For the detection of mycoplasma, the classic method is the culture in specific media and, even in Brazil, it was long suggested as a routine in cell culture laboratories[22]. However, besides being a time-consuming technique, for technical and biosecurity issues, this activity requires experienced staff and special facilities[23]. Still, since not all mycoplasmas are easily cultivable, some cases of contamination may go unnoticed. Through an electronic microscope, mycoplasmas can be seen inside cells or even on the surface of the cell membrane. But this also requires costly equipment and specialized personnel. So, in practice, other methods are routinely used. DNA labeling of the cells fixed in slides with fluorescent dyes[24] is one of these methods. In healthy cells, the dye is seen with a light fluorescence microscope only in the nucleus, but in cells contaminated by mycoplasma, the dye can be seen also dotting the cytoplasm because it also labels the DNA of this intracellular parasite (Figure 1 shows the detection of mycoplasma by DAPI). The enzyme tests, capable of showing specific mycoplasma enzymes in the cell culture supernatant, are quite specific and commercial kits are available for this purpose. Kazemiha *et al.* compared some methods for mycoplasma detection and indicated the biochemical test as a replacement to the cultivation of mycoplasmas[25]. In our laboratory, we achieved a biochemical assay equally sensitive to a PCR[2]. PCR attracts a lot of attention because it is fast, robust and highly sensitive[19] and there are commercial PCR and qPCR kits and several published methodologies. Ideally, the contamination of a cell culture by mycoplasma should be confirmed only with positive results in two of these techniques.

For the detection of viral contaminants, the classical method isolates the virus by placing an aliquot of the culture supernatant/lysate of the material to be tested on a susceptible cell culture known to be free of contaminants. Thus, viruses can be detected through morphological changes in these cells (cytopathic effect) and/or evidenced by using antiviral labeled antibodies or other serological or molecular tests. This methodology, that depends on the multiplication ability of the contaminant virus in the susceptible cells, is the only one whose positive result directly confirms the presence of viable viral particles and, for this reason, viral isolation is mandatory by the Food and Drug Administration for viral detection in FBS (9 CFR 111.47), like BVDV and Parvovirus, among others[26]. In addition to isolation, performing other tests for specific viral agents is recommended[27]. Some of these tests can detect virus or pieces of them and search for specific activities (like hemagglutination) or reactivity with specific antibodies (like virus neutralization and immunoassays). Additionally, molecular tests detect viral DNA or RNA by PCR or RT-PCR, respectively. There are methods for all of these tests in literature and commercial kits to assess the presence of several viruses. The search for these adventitious agents may require a lot of work, because each test usually detect one viral type only. For this reason, when it is not possible to use laboratory validated methods, we recommend at least the acquisition of previously tested inputs and suggest special attention to the details of the trials, including the specificity of each one and the detection limit of the

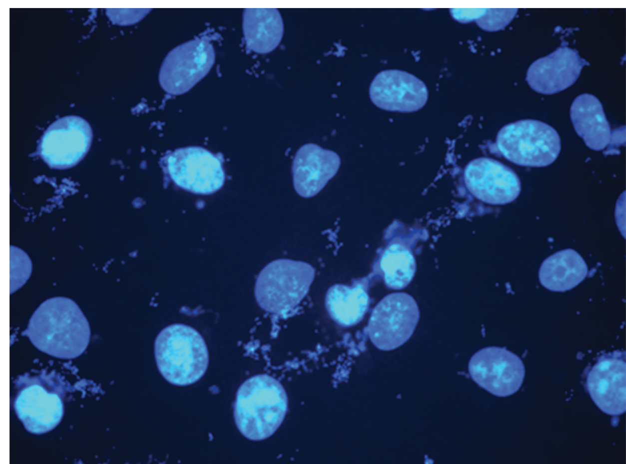

Figure 1. Cells in monolayer contaminated with mycoplasmas and stained with DAPI. Stained nuclei are observed through DNA labeling and mycoplasma DNA are also present in the cytoplasm or membrane of the cells, showing the typical "starry sky" aspect. Photo courtesy of Dr. Alberto Fraile-Ramos.

tests. This information is not usually readily available to the consumer but is essential to the evaluation and comparison of products[28]. In the case of introducing viral detection tests in the laboratory, this information is also important to choose the method, in addition to validate its use in cell cultures, since matrix effect may happen and change the efficiency of vet kits used for this activity[21].

Most methods described above search for specific contaminants. Thus, unknown or not surveyed agents will not be identified. To overcome this bias, some authors suggests metagenomics using next-generation sequencing (massive parallel sequencing) as an impartial methodology to detect viruses and other agents in sera and other animal tissues. Using Illumina methodology (Illumina, San Diego, CA, USA), Sadegui *et al.*[26] identified new viruses in FBS and stated that they could potentially contaminate cell cultures. Toohey-Kurth *et al.*[27] described the application of metagenomic methods (MiSeq, Illumina, San Diego, CA, USA) for virus detection in commercial bovine sera. 26 commercial sera from 12 independent manufacturers of the USA, Australia and New Zealand were analyzed, including 20 FBS. This study detected nine viral families and four unknown viruses. The technique was as sensitive as a RT-qPCR (reverse transcription quantitative PCR). The viral numbers varied from zero to 11 among samples and from one to 11 among suppliers, and only one product from one supplier was entirely "clean". The authors consider that these findings illustrate that relying on known virus specific test panels (although this may fulfill regulatory requirements) is inadequate to address the full range of the biological problem. In the future, metagenomics might become a qualitative and quantitative tool for quality control of serum derived biological products, serum itself and cell cultures.

While these new technologies are not available for the quality control of cell cultures and their inputs, the quality of the cells we use in the laboratory and of the cell culture products will deserve our utmost attention. Moreover, we believe that there should be a greater effort to improve the guidelines and regulations for activities that involve cell cultures.

As an example of this initiative, we observe that several protocols recommended by the Organization for Economic Cooperation and Development (OECD - www.oecd.org) for alternative methods to using animals, using cell lines, require that the they are tested for mycoplasma contamination: (i) OECD TG 129 Method - Estimation of the starting doses for acute systemic oral toxicity tests (http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2010)20&doclanguage=en) (ii) OECD TG 432 Method - In vitro 3T3 NRU phototoxicity test (http://dx.doi.org/10.1787/9789264071162-en); (iii) OECD TG 439 Method - In vitro skin irritation test (http://dx.doi.org/10.1787/9789264242845-en); (iv) OECD TG 460 Method - Fluorescein leakage test (http://dx.doi.org/10.1787/9789264185401-en); (v) OECD TG 487 Method - *In vitro* mammalian cell micronucleus test (http://dx.doi.org/10.1787/9789264091016-en); (vi) OECD TG 491 Method - Short time *in vitro* test for identifying eye damage (http://dx.doi.org/10.1787/9789264242432-en). It is worth mentioning that in Brazil these methods are recognized by the Brazilian Council for Animal Experimentation Control (Concea) through the Normative Resolutions n. 18, of September 24, 2014 and n. 31, of August 18, 2016. The publication of the Collegiate Board of Directors Resolution (RDC) n. 35, of August 7, 2015, of Anvisa (Brazilian Sanitary Surveillance Agency) formalized the acceptance of the alternative methods to animals experimentation listed in the previously mentioned resolutions. This RDC also demanded a total replacement of animal use for this kind of methodologies until 2019.

Authentication of human and non-human cell lineages

Cell lineages are the most widely used model in biomedical research and in the manufacture of biological products. The first lineage, established in 1952, was extracted from a patient with cervical cancer (Henrietta Lacks) and, in tribute to her, the lineage was named as HeLa[29]. The model soon became popular and new lineages were created, like BT-20, the first breast cancer lineage[30], and MCF-7, also a primary breast tumor lineage, however, metastatic and isolated from a pleural effusion[31].

In the late 50's, the first case of misidentification/contamination of a cell lineage was reported. It was described by Rothfels *et al.*[32] in 1958. Since then, governments and research institutes try to expose and fight this problem. In 1962, the authenticated cell bank of the American Type Culture Collection (ATCC) (www.atcc.org) was created. This institution tested all human and non-human cell cultures by karyotyping (chromosomal banding pattern) or performing isoenzyme electrophoresis (a technique that could verify cellular authenticity on a large scale[33,34,35,36].

Karyotyping was the first technique that made it possible to identify a contaminated cell lineage. It is a well-established methodology described in a harmonized guide[37] as an *in vitro* assay for testing chemicals for mammal chromosomal aberration analysis. The method assumes that each species has a unique set of chromosomes and, therefore, has been used to identify species of primary cultures. On the other hand, in the analysis of cell lines, karyotyping can be quite complicated, since lineages cultivated for long periods of time are subject to different experimental conditions and can be genetically unstable and create cell heterogeneity. In fact, karyotyping allows an overview of the genome and the identification of the original species within a huge spectrum using the same methodology. However, because karyotyping is based on the number and structure of the chromosomes, the method is extremely laborious and time-consuming. It requires at least 20 metaphase spreads, but usually between 50 to 100 are made and often it is still insensitive to detect contaminated cells. Although the standard analysis of metaphasic cells may not be sensitive enough to detect cross-contamination[38], they can determine gender, ploidy and genetic stability, but not very accurately[39]. Some automated systems that use markers for each chromosome made the technique less laborious, yet more expensive[40]. Thus, with the development of faster, more accurate and less laborious methods, the use of karyotyping for the authentication of cell lineages,

especially human ones, was drastically reduced, although it is still useful in some specific situations, besides being widely used in other applications, like diagnostics.

Briefly, isoenzyme analysis detects polymorphisms of cytosolic enzymes due to changes in their electrophoretic mobility that allows to discriminate cell lineages of different animal species[41]. However, making the correct choice of the set of enzymes to be analyzed, interpreting the results and finding commercial bits for this assay raise difficulties to this technique. Also, because techniques based on DNA analysis tend to be faster, cheaper and more sensitive than those based on proteins, this methodology was also replaced by new ones. Table 1 summarizes the main techniques used to authenticate human and non-human cells and presents a brief description of the principles and applications of each methodology.

Currently, the recommended technique for genotyping human cell lineages is the Short Tandem Repeat (STR) profile (Figure 2) focused on nine *loci* and amelogenin (sexual identification), with some variation between different databases. Today, the chosen markers are: D5S818, D13S317, D7S820, D16S539, D21S11, vWA, TH01, Amelogenin, TPOX, CSF1PO[42].

It is interesting to highlight that although STR analysis may in theory be used to identify all animal species, currently these markers in cell banks are used specifically to identify humans. Few initiatives attempt to apply these methodologies to other species[43]. As a result, little is known about the error level when identifying animal cell lineages used in the industry, including the production of recombinant proteins, vaccines, other biological products[38] and interspecies contamination. Although mice STR primers are available, its use remains a challenge since many mouse cell lineages are derived from only one individual, therefore indistinguishable. Plus, it would be necessary to develop primers for all commercial species, that is, identify polymorphic reliable STR for intraspecies discrimination, including contributions from many laboratories collecting data from STR to the same cell lineage in order to build a consensus in the identification. In conclusion, barcode assays, karyotyping by G banding and analysis of surface markers are still the chosen methods to characterize non-human cells, for example, as recently reported in a study that characterized a identification error in a mouse cell lineage, the RGC-5[44].

The STR *loci* are genome regions with nucleotide sets repeatedly sequenced. The STR varies from two to seven nucleotides per repetition unit and it is common to use STR *loci* with four or five nucleotides per repetition. The smaller this number is, the smaller the total fragment will be, which increases the probability of success to analyze degraded DNA (most forensic samples). However, STR with two or three nucleotides per repetition usually generates more artifacts that are inherent in the technique and, for this reason, the best relation between the smallest fragment with fewer artifacts are the STR with four or five repetitions[45,46].

The number of repetitions is the allele and can be the same or different between individuals of a population. There are hundreds of STR *loci* distributed in all human chromosomes. The possible number of alleles (or repetitions) is finite and each allele has a frequency within the population and the demes. Based on these characteristics, 13 STR *loci* were selected (in this case, it is common to refer to the gene *locus* as a genetic marker) to be used in human identification, paternity research and forensic sciences. As a result, a lot of empirical material was generated

Table 1. Comparison between the main techniques used in cell authenticity.

Technique	Description	Application	Ref
Chromosomal analysis/karyotyping	It involves the preparation of a metaphase spreading with chromosomal bands and staining to identify the chromosome number and markers.	Spp; Ind	(52)
Isozyme Analysis	Biochemical method to separate isoenzymes by electrophoresis; the mobility of isoenzymes may vary between individuals or species. Available kits include Authentikit gel electrophoresis system.	Spp; Ind	(53,54)
DNA fingerprint multilocus	Molecular method to detect length inside DNA minisatellites containing a variable number of tandem repeats. The analysis is made by Southern blot using DNA probes, M13 phages or oligonucleotide sequences.	Ind	(55,56)
STR Profile - Short Tandem Repeat	Molecular method to detect length variation inside DNA microsatellite regions containing a variable number of tandem repeats. The analysis is made by PCR/capillary electrophoresis. The interpretation depends on a molecular weight pattern and a specific calculation software.	Ind	(57,58)
SNP - Single Nucleotide Polymorphism	Molecular method to detect mutations in a single nucleotide. The analysis is based on the identification of a base among two known options for each *locus*. While in STR the allele is the number of repetitions of a set of nucleotides, here the allele is a nucleotide (for example, for the 55009062 position of chromosome 7 there is a SNP whose alleles are G and T and the frequency of T is approximately 25%, as described in https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=712829). The great advantage of this bi-allelic marker is the ability to work with highly degraded samples. The disadvantage is the need for a much higher number of *loci* than the STR.		(59,60)
DNA Barcode	Involves sequencing a fragment of cytochrome C oxidase subunit 1, a mitochondrial gene. The analysis consists in aligning different sequences in the same position. The differences are recorded in a group multivariate analysis. The number of sequences in public databases reaches millions. DNA Barcode has shown to be a practical technique to distinguish a large number of species, practically any species that has mitochondria.	Spp	(61,62)

Spp = species; Ind = individual or lineage; ref = references.
STR: Short Tandem Repeat; SNP: Single Nucleotide Polymorphism; PCR: polymerase chain reaction.

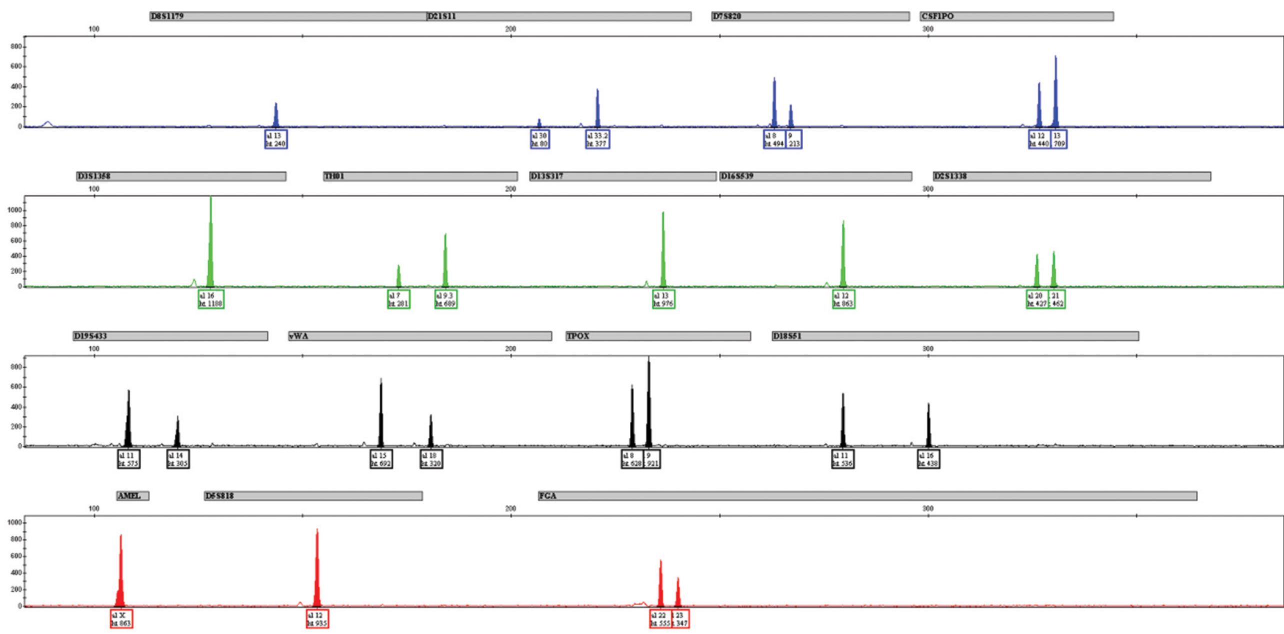

Figure 2. STR profile. The figure represents an electropherogram interpreted by a specific software. The gray rectangles show the name of the marker and the range of known alleles. The peaks represent the alleles (number of repetitions). The more on the right, the larger the fragment and, therefore, the number of repetitions. Each marker (gene *locus*) may have a peak (homozygous) or two peaks (heterozygote). Since humans are diploids, one peak represents the allele present on the chromosome inherited from the father and the other from the mother. Under normal conditions, the marker having only one peak means that both chromosomes have the same allele. For markers with two peaks, in some of them, it is possible to notice that there is a greater height difference (allelic imbalance). For healthy humans, a difference of up to 30% is accepted, however the cells immortalized through passages can show this difference as a result of genetic degeneration. In addition, the presence of three or four peaks in one or two markers is not a rare event.

Table 2. Major cell banks with genetic profile banks.

Cell Banks	Country	URL
ATCC	USA	http://www.atcc.org/
Cell Bank of Australia	Australia	http://www.cellbankaustralia.com/
DSMZ	Germany	http://www.dsmz.de/
ECACC	United Kingdom	http://www.hpacultures.org.uk/collections/ecacc.jsp
ICLC	Italy	http://www.iclc.it/
JCRB	Japan	http://cellbank.nibio.go.jp/
RIKEN	Japan	http://www.brc.riken.go.jp/lab/cell/english/guide.shtml

ATCC: American Type Culture Collection; DSMZ: *Deutsche Sammlung von Mikroorganismen und Zellkulturen*; ECACC: The European Collection of Cell Cultures; ICLC: Interlab Cell Line Collection; JCRB: Japanese Collection of Research Bioresources Cell Bank; RIKEN: Designated National Research and Development Institute.

and made available by universities, research centers, forensic experts, and private companies[47,48,49].

This investment improved the development of equipment, procedures, validation and, consequently, the maturation of the knowledge on the technique which, in turn, became more robust, reproducible, eligible to automation, relatively cheap and with several validated commercial kits available. Furthermore, a great technical knowledge is not needed to perform the test. Accordingly, this methodology was chosen by the scientific community and implemented in the ANSI/ATCC SDOW ASN-0002: 2011 norm[33,35,42,50] that presents a comprehensible process for the test execution, including requirements related to the test quality.

Regardless of the kit used, the allele will always be the same for one marker in one individual and, therefore, eligible to comparison with different databases (Table 2). Besides the possibility to search a certain profile by the lineage name and compare it manually, the websoftware CLIMA[51] can search for STR profiles using the alleles. The CLIMA (http://bioinformatics.hsanmartino.it/clima2/index_test.php) searches amongst 5.450 available profiles in different databases and returns the lineages at least 60% similar, the most similar on the top of the list[51].

Immortalized cell lineages may undergo different genetic changes throughout their passages and this may affect their STR

profile. The *International Cell Line Authentication Committee* (ICLAC - iclac.org) is an international committee for cellular authenticity and the ANSI/ATCC SDOW ASN-0002 norm[42] suggests that an identity agreement between the reference profile and the questioned profile of up to 80% still indicates authenticity, that is, that the cells proceed from the same donor. This is the standard adopted worldwide that allows cross comparability of results. Although well established, there are few studies on contamination surveys.

A study estimated that from 18% to 36% of all cell cultures used in the world have authenticity errors and intra or interspecific contamination[63,64]. In Brazil, data suggest that 12% (11/91) of the cell cultures are contaminated or poorly identified[65], while the *Deutsche Sammlung von Mikroorganismen und Zellkulturenrc* (DSMZ) reported 17.9% (45/252)[63]. Still, the Iranian Cell Bank found error in 18.9% of the cultures (10/53)[66], the Bioresource Collection and Research Center of China (BCRCC) in 16.3% (17/104)[67], and the China Infrastructure of Cell Line Resource (CICR) in 20.7% (100/482)[68].

It is interesting to notice that although there is an ICLAC initiative to publish misidentified cell lineages (including 24 non-human cell lineages amongst the 488 lineages having identity errors)[69], the cell authentication guide offered by the committee currently contemplates only human cells[69]. Therefore, it is highly possible that these data is underreported and, even though regulatory agencies also demand the authentication of non-human cell lineages, there is no consensus as to the best method. As a result, methods hardly used in laboratory routines, like biochemical, immunological and cytogenetic (table 1) methods are implemented.

Hence, DNA Barcode has been suggested as the method scientists should choose to determine the species of a cell lineage. It analyzes a conserved mitochondrial gene with intraspecies sequence variation. It enables the development of a PCR based vertebrate and invertebrate speciation test[61,70]. The analysis of the cytochrome C oxidase I (COI) gene, a fragment of approximately 700 base pairs (bp), is extensively used to identify species, cell lineages, food and other animal products, forensic samples, among others. The method was created through an association between museums that needed a more reliable method to identify species, in addition to the extensive morphological analysis[71]. Later, there was intense interest of regulatory agencies because of the growing need of the market to inspect adulteration and fraud in food, mainly. DNA Barcode is a very rich tool with great potential because it is relatively simple, fast and can successfully replace more laborious and less reproducible techniques like isoenzyme biochemical typing or karyotyping. Considering that the publication of the ANSI/ATCC ASN-0002 norm in 2011 led cell repositories all over the world to perform fragment analysis for identification of human cells, one would expected that, with the publication of ANSI/ATCC ASN-0003 norm of 2015 (https://webstore.ansi.org/RecordDetail.aspx?sku=ANSI%2fATCC+ASN-0003-2015), the method of DNA Barcode would become widespread and widely used to identify non-human cell lineages. This would reduce the large ignorance about interspecies contamination in cell lineages.

The use of contaminated cell lineages often leads to erroneous and non-reproducible results. Publications with these data cost thousands of dollars, generate mistakes that could take years to be clarified and result in a significant delay in science advance. For example, Dirks *et al.*[72] showed that the endothelial cell lineage ECV-304 had become a subclone of T24 lineage, bladder carcinoma cells, probably through contamination in the original laboratory[44]. Despite this, more than 500 studies were published after the publication of this problem[73]. The validity of the results is questionable and certainly not comparable.

The issue becomes even more critical when the financial impacts are considered. In the United States[15], a study estimated that at least 28 billion dollars are spent on non-reproducible biomedical research. Poorly identified or contaminated cell lineages are listed as one of the factors that contribute to this scenario. Although a STR analysis can identify a misidentification or contamination and cost relatively little (approximately 200 dollars per sample), only a third of laboratories tests their lineages regularly[74]. Figure 3 illustrates a contaminated profile. Compared to Figure 1, it is clear that the cell sample had more than one type. Freedman *et al.*[16] properly exemplified the point: an academic researcher funded by the National Institute of Health (NIH) receives on average 450 thousand dollars. It would cost only 0.2% of it to validate the identity of the purchased cells and the ones in stock. In total, NIH invests 3.7 billion dollars in research using lineages or cell cultures. A quarter of these projects use cell lineages with some type of problem. This represents 750 million dollars that could promote research progress and development of new treatments for diseases[16].

Nardone[35] and Nelsson-Rees *et al.*[36] are considered the two major references in the quest to eradicate the use of contaminated or not authentic cells and proposed, among other things, that major scientific journals and even funding agencies should require confirmation of the authenticity of the cultures used in the projects/articles. Although this practice was not fully adopted, it had some positive impact, especially in the world culture of contamination verification. Capes-Davis *et al.*[75], however, chose prophylaxis and searched for the main sources of errors: incorrect inventory, labeling, typographical errors, names partially faded, simultaneous handling of different lineages and use of the same pipette in different cultures[75].

Even though the STR technique is routinely used in many genetic, molecular and cell biology laboratories, in Brazil, only the Tissue Bioengineering Laboratory (Labio, from the Brazilian Institute of Metrology, Quality and Technology, Inmetro) offers it as a standardized service to the scientific community, industry, research and technology centers. Despite this, less than 10 institutions have requested the service so far and, among these, less than five do it frequently. Except for two, all institutions are in the Southeastern region of Brazil. In the search for a culture change, courses of authenticity and purity will be offered to the laboratories of the Brazilian Network of Alternative Methods (Renama).

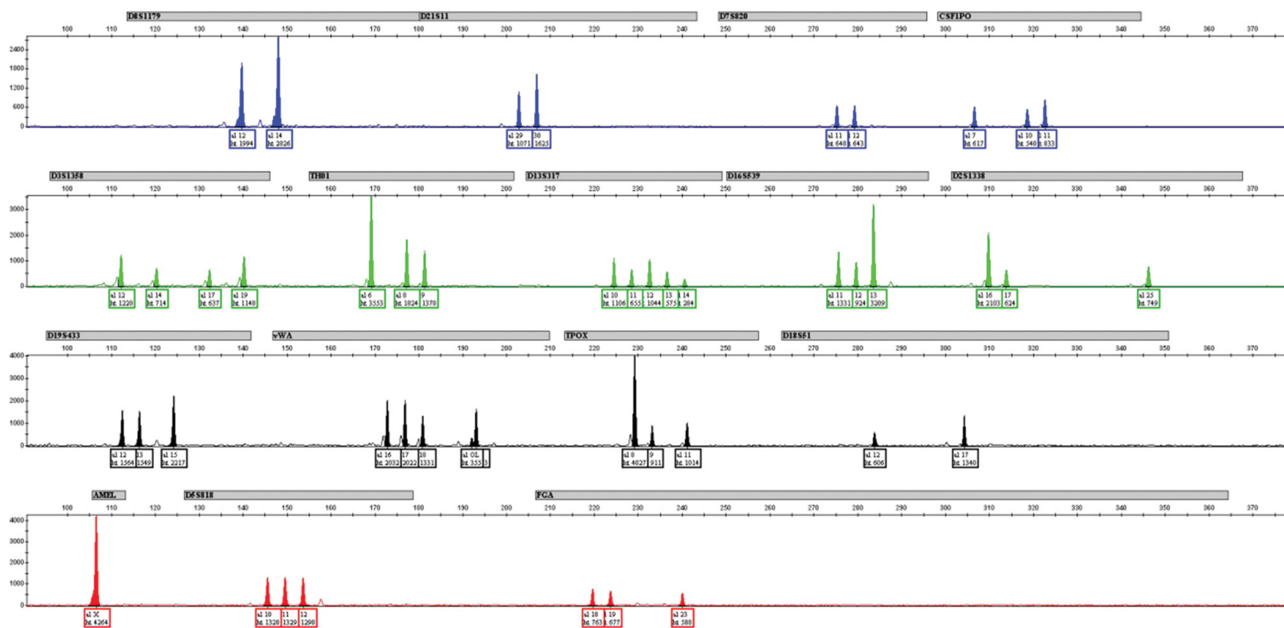

Figure 3. STR profile showing contamination. The identification of the DNA mixture of two or more individuals is indisputable. As explained before, each marker should present a maximum of two peaks (humans are diploid). Exceptionally, a trisomy is accepted in one or two markers. In the image, practically all markers show trisomies or tetrasomies, that is, three to four peaks. It indicates cross contamination between two or more individuals (cell lineages). The allelic imbalance, that is, the difference in height between two peaks within the same marker, indicates that that an allele is receiving a greater contribution than the other(s).

Despite the efforts of different groups to draw attention to the question of authenticity of cell cultures, dating back more than three decades, even today we have some disturbing numbers[68], mainly from countries that took more time to acquire the habits of checking their lineages. On the other hand, as something positive, cell authenticity and purity are no longer a concern only in the United States-Europe-Japan axis. It is now a global movement of governments, companies and researchers for more reproducible and better quality science.

Current overview of cell quality control performed by biological collections

In 2014, Geraghty *et al.*[76] suggested guidelines for the use of cell lineages in biomedical research. Among these, the first recommendation was that the lineages of a study should be acquired from a recognized biological collection. In this sense, it is a consensus among several international collections that it is important to perform STR human genetic profile identification tests and evaluate mycoplasma contamination. Depending on the available infrastructure or investment in other techniques, viral contamination analyses are also available. Many cell banks, in addition to offering the distribution of tested lineages, also offer quality control services in their portfolios, as described in Table 3. In this way, the supply of cell cultures with aggregate quality increased considerably and their acquisition from biological collections that offer them after performing the proper authenticity and purity tests is the gold standard for the implementation of *in vitro* tests that use them with industrial or research purposes.

CONCLUSIONS

Considering all the investment applied in scientific research worldwide, the development of new methodologies that are alternatives to the use of animals and the critical consensus that the concept of quality must also be applied in the tools that use cell cultures in their test models, we conclude that any laboratory must guarantee the purity and authenticity of their lineages.

With the formal acceptance of Anvisa protocols recognized by Concea, several laboratories may provide *in vitro* toxicological analysis. In this sense, the OECD Guidelines are very clear as to the quality control of the lineages, like the record of the periodic control of mycoplasma contamination, for example. If it is a human lineage, we recommend to identify the genetic profile through STR testing. For non-human lineages, the barcode emerges as a possible technique to identify, at least, the species, that can always be associated with a morphological evaluation and the original literature data about its isolation.

Furthermore, in tumor cell studies, whose vast majority is performed with human cells, several international journals already require cellular identity tests for publishing articles. Thus, Inmetro, as the Central Laboratory of Renama, has been engaged in the development and implantation of cell purity and authenticity methodologies so that the training and/or offering of this type of service to Brazilian laboratories and research centers becomes possible. The goal is to increase Brazilian data reliability and reproducibility.

Table 3. Demonstrative table of quality control of distributed lineages and other services offered by international biological collections.

Biological Collection	Quality control of the collection cell lineages	Services Offered
ATCC (USA)	• STR profile (human lineages) • Sterility tests for bacteria, fungi and mycoplasmas • Viral detection tests for HBV, cytomegalovirus, HIV, EBV, HPV	• Analysis of human genetic profile through STR • Mycoplasma contamination analysis through culture • Mycoplasma contamination analysis through fluorescence • Distribution of cell lineages
Cell Bank of Australia	• The bank commercializes lineages of the ECACC, submitted to original control. However, in exclusive lineages of its collection, it controls the following: • STR profile (human lineages) • Barcode for species identification • Mycoplasma sterility tests	• Analysis of human genetic profile through STR • Species identification for non-human lineages through Barcode • Mycoplasma contamination analysis through PCR • Mycoplasma contamination analysis through Bioluminescence • Distribution of cell lineages
DSMZ (Germany)	• Mycoplasma sterility tests • Cytoskeleton and membrane proteins immunostaining • DNA fingerprinting • Barcode for species identification • Karyotyping • Analysis of viral contamination by HBV, HCV, EBV, HHV-8, HHV-4, HIV-1, HIV-2, HTLV 1 and 2, HPV, SMRV, XMRV	• Analysis of human genetic profile through STR • Online STR profile analysis obtained by customers • Species identification for non-human lineages through Barcode • Mycoplasma contamination analysis through PCR • Mycoplasma contamination analysis through culture • Decontamination service of mycoplasma contaminated lineages • Viral Detection Service: HBV, HCV, EBV, HHV-8, HHV-4, HIV-1, HIV-2, HTLV 1 and 2, HPV, SMRV, XMRV • Distribution of cell lineages
ECACC (United Kingdom)	• Human genetic profile analysis through STR • Online STR profile analysis obtained by customers • Species identification for non-human lineages through Barcode • Mycoplasma contamination analysis through PCR • Mycoplasma contamination analysis through culture • Microbiological contamination analysis and bacteria and fungi identification	• Human genetic profile analysis through STR • Online STR profile analysis obtained by customers • Species identification for non-human lineages through Barcode • Mycoplasma contamination analysis through PCR • Mycoplasma contamination analysis through culture • Mycoplasma contamination analysis through fluorescence • Microbiological contamination analysis and bacteria and fungi identification
ICLC (Italy)	• Species identification through isoenzymes • Species identification through PCR using specific oligonucleotides • Identification of human lineage through STR • Mycoplasma sterility tests	• Mycoplasma contamination analysis through PCR • Analysis of mycoplasma contamination through biochemical assays • Mycoplasma contamination analysis by fluorescence • Distribution of cell lineages
RIKEN (Japan)	• Human genetic profile analysis through STR • Species identification through isoenzymes and PCR • Identification of the mouse lineage by SSLP • Identification of the viral contaminants HBV (from liver), HCV (liver), HIV (blood), HTLV-1 (blood) and EBV (blood and tumors) • Mycoplasma sterility test through PCR and isoenzymes	• Distribution of cell lineages
BCRJ (Brazil)	• Human genetic profile analysis through STR • Mycoplasma sterility test through bioluminescence, PCR and/or fluorescence • Bacteria and fungi sterility microbiological test	• Mycoplasma contamination tests through bioluminescence or PCR • Distribution of cell lineages

STR: Short Tandem Repeat; SNP: Single Nucleotide Polymorphism; PCR: polymerase chain reaction; ATCC: American Type Culture Collection; DSMZ: *Deutsche Sammlung von Mikroorganismen und Zellkulturen*; ECACC: The European Collection of Cell Cultures; ICLC: Interlab Cell Line Collection; RIKEN: Designated National Research and Development Institute; BCRJ: Rio de Janeiro Cell Bank

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Conflict of Interest

Authors have no potential conflict of interest to declare, related to this study's political or financial peers and institutions.

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