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ORIGINAL ARTICLE

National production of certified reference fungal cultures

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Abstract Reference fungal cultures (RFCs) are essential for the internal quality control of laboratories. The production of these cultures requires standardized procedures (IRAM 14950:2016 and ISO 17034:2016 standards) carried out by a recognized and accredited laboratory. The aim of this work was to produce RFC in paper disks of autochthonous strains, characterized by two, homogeneous and stable reference methods traceable at species level. RFC were produced using 14 regional species (7 yeasts and 7 filamentous fungi) from the fungal culture collection (DMic). Paper disks were impregnated with a culture suspension, dried and packed. Homogeneity, viability, identity and purity were verified. Short- and long-term stability at different temperatures and storage times were studied. Characterization of each strain allowed to confirm its identity and to ensure its traceability at international level. Produced batches were homogeneous and stable at $-20 \pm 5\,^{\circ}C$ for 30 months. This method of production was adequate to produce homogeneous and stable RFC with phenotypic and genotypic characteristics correctly defined and internationally traceable. Standardized procedures were developed for the production of certified RFC that could be transferred to other microorganisms. Providing RFC that represent regional strains allows laboratories to produce more reliable results with a favorable impact on medical diagnosis, the environment or the food industry.

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PALABRAS CLAVE
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Colección de cultivos

Producción nacional de cultivos fúngicos de referencia certificados

Resumen Los cultivos microbianos de referencia (CR) son imprescindibles para el control de calidad interno de los laboratorios. Asegurar su producción requiere de procedimientos estandarizados (IRAM 14950:2016 e ISO 17034:2016) realizados en un laboratorio reconocido y acreditado. El objetivo de este estudio fue producir cultivos fúngicos de referencia en discos de papel, a partir de un panel de cultivos autóctonos caracterizados por dos métodos de referencia, trazables a nivel taxonómico de especie, homogéneos y estables. Se produjeron CR de 14 especies circulantes en Argentina (7 de levaduras y 7 de hongos miceliales), depositadas en la colección de hongos de interés médico (DMic). Los discos de papel fueron embebidos con una suspensión del cultivo por producir, secados y envasados. Se verificó la homogeneidad, viabilidad, identidad y pureza de cada lote. Se evaluó la estabilidad a corto y largo plazo a distintas temperaturas y tiempos de almacenamiento. La caracterización de cada CR nos permitió confirmar su identidad y asegurar su trazabilidad a nivel internacional. Los lotes producidos fueron homogéneos y estables durante 30 meses conservados a $-20 \pm 5\,^{\circ}C$. Este método resultó adecuado para producir CR homogéneos y estables, con características fenotípicas y genotípicas correctamente definidas y trazables a nivel internacional. Los procedimientos estandarizados desarrollados en este trabajo pueden ser transferidos para producir CR certificados de otros microorganismos. La provisión de CR que represente cepas regionales permite a los laboratorios producir resultados más confiables con un impacto favorable en el diagnóstico médico, los estudios ambientales y la industria alimenticia.

Introduction

Microbiology laboratories require the use of microbiological reference materials produced under international standard procedures to ensure that reported results are comparable and reproducible. Reference fungal cultures (RFCs) that come from culture collection centers with international recognition constitute such references. Acquiring this type of material is conditioned by RFC prices and the cost of import customs clearance formalities, especially for public health laboratories in developing countries. Moreover, additional difficulties arise as some of these agents are considered potential agents of bioterrorism and due to this fact; its distribution has been restricted. In addition, many commercially available RFC may not represent local circulating strains. To face this problem, there is a need to establish requirements for the development and production of certified reference microbial cultures.

Reliable, reproducible reference materials that guarantee their identity over time allow the laboratory to make decisions based on facts that ensure the quality of the services it provides. These reference materials are essential for proficiency testing programs with reference quantity values, internal laboratory quality controls, validation of methods and equipment and training of personnel.

Based on this situation, requirements for reference microbial culture production were defined in Argentina through the publication of IRAM standard 14950:1999 ''*Reference microbial cultures. Requirements for the competence of reference microbial culture producers*'' which was updated in 2016[3]. Its aim is to ensure traceability of the microorganism at the level of genus and species and to agree on a new definition for a reference strain, as opposed to the concepts that define it as a microorganism obtained directly from a culture collection, which leaves aside the property value[10]. IRAM 14950:2016 standard defines a reference strain as a population of microbial cells with established phenotypic and genotypic characteristics. The implementation of this standard will allow RFC producers to guarantee the qualitative properties of this material as a whole[3].

Developing local reference microbial cultures that represent regional strains will allow microbiology laboratories to produce more reliable results with a favorable impact on medical diagnosis, the environment or the food industry. The production of these cultures requires standardized characterization procedures, homogeneity and short- and long-term stability assessment carried out by a recognized and accredited laboratory which implements a quality management system based on IRAM 14950:2016 and ISO 17034:2016 standards[3,5]. Reference laboratories from regional countries that preserve microbial cultures could assume the responsibility and challenge of producing such reference materials by implementing these standards in order to be accredited as competent producers at national and international level.

The aim of the present work is to produce reference microbial cultures embedded and dried in paper disks from a panel of autochthonous strains. These cultures will be characterized by two different reference methods, will be traceable to genus and species identification and will be characterized with respect to homogeneity and stability in compliance with standardized procedures[3,5].

Table 1 Growth media and culture conditions for preparation and preservation of cell bank microorganisms[4,9]

Microorganism	Culture media (in-house)	Crioprotectant	Solvent used for production run
Cryptococcus spp. and *Candida* spp.	YM agar	20% Glycerol	Distilled water
Aspergillus spp.	CYA	10% Glycerol, 0.1% Tween-80	0.1% Tween-80
Trichophyton spp.	LAC	10% Glycerol	Distilled water
P. variotii and *Fusarium* spp.	PDA	10% Glycerol	Distilled water
R. arrhizus	PDA	10% Glycerol, 0.1% Tween-80	0.1% Tween-80

Cryptococcus spp., *Candida* spp. and other yeasts were incubated at 27 ± 3 °C for 72 h and the remaining microorganisms were allowed to grow until observing characteristic morphology.
YM agar (yeast extract–malt extract–peptone–glucose): yeast extract: USB Corporation, Cleveland, Ohio, USA; malt extract: Oxoid Ltd, Hampshire, UK; peptone: BD, Becton, Dickinson and Co., Maryland, USA; glucose: Anedra Research AG S.A., Troncos del Talar, Buenos Aires, Argentina; Bacteriological Agar (Agar No. 1): Oxoid Ltd, Hampshire, UK.
CYA (Czapek yeast extract agar): sucrose: Laboratorios Britania S.A., City of Buenos Aires, Argentina; yeast extract: USB Corporation, Cleveland, Ohio, USA; dipotassium hydrogen phosphate: Merck, Darmstadt, Germany; sodium nitrate: Mallinckrodt Chemicals Works, New York, NY, USA; potassium chloride: Sigma-Aldrich Corporation, St. Louis, Missouri, USA; magnesium sulfate: Mallinckrodt Chemicals Works, New York, NY, USA; ferrous sulfate: Sigma-Aldrich Corporation, St. Louis, Missouri, USA; zinc sulfate: Merck, Darmstadt, Germany; copper sulphate: Fluka Chemie AG, Buchs, Switzerland; Bacteriological Agar (Agar No. 1).
LAC (Borelli's Lactrimel agar for dermatophytes): Bacteriological Agar (Agar No. 1); wheat flour; milk; honey.
PDA (potato dextrose agar): glucose; Bacteriological Agar (Agar No. 1); potato.
Tween 80: Biopack, Zárate, Province of Buenos Aires, Argentina.
Glycerol: Biopack, Zárate, Province of Buenos Aires, Argentina.

Materials and methods

Project design

Production of RFC was designed by first identifying the most relevant stages of the production process, such as selection of candidate strains, verification of their identity, viability and purity, master cell bank generation, production planning, assessment of reference material homogeneity and short and long-term stability, characterization and value assignment of reference material (taxonomic classification, genus and species). Each of the production stages will be discussed below.

Reference material, selection of candidate strains

Local fungal cultures from the Fungal Culture Collection of Biomedical Interest (DMic) were selected based on phenotypic and genotypic characteristics for species identification. The DMic is a member of the World Federation of Culture Collection (WFCC) and is registered with the World Data Center for Microorganisms (WDCM) under number 1169. These cultures were stored at −70 ± 10 °C using glycerol as cryoprotectant as detailed in Table 1.

The microbial panel was comprised of 18 selected strains: *Candida albicans* DMic 113874, *Candida krusei* DMic 134341, *Candida parapsilosis sensu stricto* DMic 113911, *Candida tropicalis* DMic 113920, *Candida glabrata sensu stricto* DMic 113884, *Cryptococcus neoformans* (genotype VNI) DMic 083456, *Cryptococcus gattii* (genotype VGIII) DMic 073122, *Aspergillus fumigatus* DMic 093603, *Aspergillus flavus* DMic 113984, *Aspergillus tubingensis* DMic 84052, *Aspergillus niger* DMic 103781, *Aspergillus terreus* DMic 134330, *Paecilomyces variotii* DMic 114056, *Fusarium solani* DMic 114020, *Fusarium oxysporum* DMic 134373, *Trichophyton mentagrophytes* DMic 134312, *Trichophyton rubrum* DMic 134313 and *Rhizopus arrhizus* DMic 124205.

Verification of the identity, viability and purity of panel strains

For each strain, one vial stored at −70 ± 10 °C was thawed in a water bath at 20–30 °C for 5 min and homogenized by manual shaking. To verify their presumptive identity, viability and purity, 50–100 μl of each vial were inoculated into different standard culture media as indicated in Table 2 and incubated at 27 ± 3 °C.

Candida and *Cryptococcus* species identity was verified using reference methods based on phenotypic characteristics[9]; mycelial fungi identity of genera *Trichophyton*, *Aspergillus*, *Paecilomyces*, *Fusarium* and *Rhizopus* was carried out using macro and micro morphology-based methods[4].

Master cell bank generation and quality control

The microbial cultures verified above were subcultured in the media and under the conditions specified in Table 1. For long-term storage, fungal propagules were preserved using the cryoprotectants indicated in Table 1 and stored at −70 ± 10 °C or liquid nitrogen. The master cell bank comprised cultures considered the starting material for all production lots, which were defined as ''reference stock'' and stored in liquid nitrogen and cultures considered as seeds for the production run, which were defined as ''stock culture'' and stored at −70 ± 10 °C. Quality control of the batches corresponding to the generation of the master cell bank was carried out on the first and seventh days after completion of the process of preservation of each lot. Viability, purity and identity were assessed according to the acceptance criteria described in Table 2.

Table 2 Assessment of viability, purity and presumptive identity

Microorganism	Culture media	Incubation period	Acceptance criteria
C. albicans	CHROMagar Candida®	1–3 days	Growth; green-colored colonies with creamy consistency. Absence of contaminants
C. tropicalis	CHROMagar Candida®	1–3 days	Growth; blue-colored colonies with creamy consistency. Absence of contaminants
C. krusei	CHROMagar Candida®	1–3 days	Growth; pink-colored colonies with dry consistency. Absence of contaminants
C. parapsilosis	CHROMagar Candida®	1–3 days	Growth; pink-colored colonies. Absence of contaminants
	Malt extract	1–7 days	Ovoid cells with multipolar budding. Cylindrical cells might be present. Pseudohyphal development
C. glabrata	CHROMagar Candida®	1–3 days	Growth; cream to mauve-colored colonies. Absence of contaminants.
	Rapid trehalose test	Every 15 min, up to 2 h	Positive: trehalose assimilation
C. neoformans	CAA	1–5 days	Growth; brown-colored colonies. Absence of contaminants
	CGB	1–5 days	No growth. Medium color remained yellow-green.
C. gattii	CAA	1–5 days	Growth; brown-colored colonies. Absence of contaminants
	CGB	1–5 days	Growth; medium color change from yellow-green to blue.
Aspergillus spp.	CYA and PDA	3–7 days[a]	Colony growth on CYA. Absence of microbial contamination on CYA and on PDA
P. variotii, *R. arrhizus* and *Fusarium* spp.	PDA	3–14 days[a]	Colony growth on PDA. Absence of microbial contamination
T. mentagrophytes and *T. rubrum*	DTM, LAC and PDA	7–14 days[a]	Colony growth on LAC and on DTM. Absence of microbial contamination on LAC and on PDA

[a] Minimum incubation period required for the microbiological culture to develop specific morphological features at 27 ± 3 °C.
CHROMagar Candida™: Trademark by Dr. A. Rambach, Paris, France.
Rapid trehalose test: Rosco Diatabs™, Taastrup, Denmark.
CAA (caffeic acid agar): Corn Meal Agar: BD, Becton, Dickinson and Co., Maryland, USA; Tween 80%; caffeic acid: MP Biomedicals, Illkirch, France.
CGB agar (L-canavanine glycine bromothymol blue agar): agar; L-canavanine sulfate: Sigma-Aldrich Corporation, St. Louis, Missouri, USA; glycine: GIBCO BRL-Life Technologies, Inc., Gaithersburg, Maryland, USA; potassium dihydrogen phosphate: Sigma-Aldrich Corporation, St. Louis, Missouri, USA; magnesium sulfate: Sigma-Aldrich Corporation, St. Louis, Missouri, USA; thiamine HCL: Sigma-Aldrich Corporation, St. Louis, Missouri, USA; bromothymol blue: Mallinckrodt Chemicals Works, New York, NY, USA.
CYA: Czapek yeast extract agar.
PDA: potato dextrose agar.
DTM (Dermatophyte Test Medium modified with chloramphenicol and cycloheximide): BD, Becton, Dickinson and Co., Maryland, USA.
LAC: Borelli's Lactrimel agar for dermatophytes.

Microbial batch production on paper disks

RFC were subcultured in the media and under the conditions specified in Table 1. Cells were then resuspended in distilled water or a Tween-80 solution as appropriate (Table 1) and homogenized. In the case of mycelial fungi, the concentration was adjusted to a minimum value of 10^6 propagules/ml using the Neubauer chamber cell counting technique. In the case of yeast, cell count density was adjusted to that of a McFarland Standard 3 and corroborated with a spectrophotometer (Jenway 6320D, Staffordshire, UK) at a wavelength of 530 nm[11].

To verify the concentration of viable colony forming units (CFU) serial 1/10 dilutions were prepared from the inoculant suspension and 100 μl were seeded onto Sabouraud Dextrose Agar (SDA) produced in-house (peptone: BD, Becton, Dickinson and Co., Maryland, USA; glucose: Anedra Research AG S.A., Troncos del Talar, Buenos Aires, Argentina; Bacteriological Agar (Agar No. 1): Oxoid Ltd, Hampshire, UK) containing plates in duplicate. To determine the amount of CFU/ml, plates were incubated for 24–72 h and the number of colonies was multiplied by the dilution factor of the plate counted.

Three milliliters of the suspension of known concentration was poured onto 100 sterile Whatman grade 1, 0.7 cm diameter paper disks distributed in a 90 mm Petri dish taking care that disks did not overlap and were covered completely by the cell suspension. Disks were allowed to impregnate for 5–10 min; then were placed in a 140 mm Petri dish under aseptic conditions and allowed to dry at 27 ± 3 °C for

48–72 h. Once dry, three disk units per vial were packed. Labeled vials were stored at 5 ± 3 °C or at -20 ± 5 °C.

Lot homogeneity control

Homogeneity of each batch was evaluated using the following formula for the random selection of vials: $\sqrt[3]{n}$, where n is the number of randomly selected vials. Viability, purity and identity were assessed for each vial as described in Table 2.

Acceptance criteria were as follows: (a) if 100% of the vials complied with the verification criteria described in Table 2, the batch was considered homogeneous; (b) if the number of compliant vials was between 90% and 100%, a second sampling was conducted using the same formula on the remaining vials. In this case, only if 100% of the vials were compliant, the batch was considered homogeneous; otherwise, the batch was discarded.

Short-term stability control of produced lot

Once homogeneity of the batches was confirmed, 10 vials were randomly selected. Two vials were tested for each of the following incubation temperatures: -70 ± 10 °C; 5 ± 3 °C; 27 ± 3 °C; 37 ± 2 °C and 55 ± 2 °C; furthermore, vials were tested for the following incubation periods: 1, 7 and 14 days. In both cases, 2 disks per temperature/time were assessed. If 100% of the disks complied with the verification criteria described in Table 2, the batch was considered stable. Through this study, which was carried out only once, optimal vial preservation conditions for transportation were established.

Characterization and value assignment

Two vials were randomly selected and one disk was taken from each vial. Cultures were recovered from these paper disks and the phenotypic and genotypic identification of microorganisms was assessed by two reference methods. Genotypic identification of fungal species was performed by sequencing the ITS1-5.8S-ITS2 region for *Candida*, *Cryptococcus*, *Rhizopus*, *Paecilomyces* and *Trichophyton* genera; in the case of *Aspergillus*, Beta Tubulin and Calmodulin genes were sequenced using Genetic Analyzer software (Hitachi 3500, Applied Biosystems, Foster City, California, USA). The sequences obtained were compared with sequences from the GenBank database of different fungal strains through the *National Center for Biotechnology Information* (NCBI) and the *Centraalbureau voor Schimmelcultures* (CBS)[8]. A culture was considered of reference when 100% of the results obtained were concordant by both methods. The value assigned to each reference microbial culture corresponds to the identity of the microorganism, *i.e.* its phenotypic and genotypic characteristics, which constitute a qualitative attribute. These characteristics were defined at the species level using recognized methodologies.

Long-term stability control of produced lots

Twelve vials were randomly selected and 4 vials were tested for each of the following incubation temperatures: -20 ± 5 °C, 5 ± 3 °C and 27 ± 3 °C; in addition, vials were tested for the following incubation periods: 1, 6, 12, 18, 24 and 30 months. If 100% of the disks complied with the verification criteria described in Table 2, the batch was considered stable. This study established the validity period of the lot and label integrity. In addition, it allowed to set the monitoring frequency of successive production batches.

Availability of reference microbial cultures and supply to the user

Each batch of production was accompanied by a Batch Certificate of Analysis, a Material Safety Data Sheet of each strain and easy-to-follow instructions for the user. For transportation, vials were placed in a triple pack that complied with the current regulations for the transport of infectious materials and were shipped at room temperature[7].

User evaluation of RFC

RFC lots of *C. krusei* and *C. neoformans* were sent to laboratories participating in the National Quality Control Program in Mycology (Programa Nacional de Control de Calidad en Micología, in Spanish PNCCM) of the Mycology Department of the National Institute of Infectious Diseases (Instituto Nacional de Enfermedades Infecciosas, INEI) ''Dr. Carlos G. Malbrán'' – ANLIS, in order to assess their identity and stability during transportation in clinical laboratories of different technical capabilities distributed among the 24 districts of the country.

Results

Considering the selected panel, it was possible to achieve the production of 14 out of 18 of the local isolates: *C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, *C. tropicalis*, *C. neoformans*, *C. gattii*, *A. flavus*, *A. fumigatus*, *A. tubingensis*, *A. niger*, *A. terreus*, *P. variotii* and *R. arrhizus*. It was not possible to obtain enough inoculum to prepare paper disks containing *T. mentagrophytes* and *T. rubrum*. *F. solani* and *F. oxysporum* failed to survive the dehydration process during disk drying. Homogeneity tests showed that all batches produced met established criteria. Identity, viability and purity results of short-term stability tests were satisfactory in 100% of the vials evaluated when stored at -70 ± 10 °C and 5 ± 3 °C for 14 days. Results were variable at other temperatures as shown in Table 3.

Long-term stability test results are shown in Table 4. All batches stored at -20 ± 5 °C for 30 months and at 5 ± 3 °C for 12 months met acceptance criteria.

In all produced batches, culture characterization allowed to confirm the identity of each strain by two different reference methods and to ensure its traceability at international level. Recovered culture phenotypic and

Table 3 Short-term stability

Microorganism	DMic number[a]	Days														
		1	7	14	1	7	14	1	7	14	1	7	14	1	7	14
		−70 ± 10 °C			5 ± 3 °C			27 ± 3 °C			37 ± 2 °C			55 ± 2 °C		
C. krusei	13434	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
C. neoformans	08345	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
A. niger	10378	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
A. terreus	13433	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
A. tubingensis	84052	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
R. arrhizus	12420	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
C. gattii	07312	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1
C. albicans	11387	2	2	2	2	2	2	2	2	2	2	2	2	0	0	0
C. parapsilosis	11391	2	2	2	2	2	2	2	2	2	2	2	2	0	0	0
A. flavus	11398	2	2	2	2	2	2	2	2	2	2	0	0	2	0	0
A. fumigatus	09360	2	2	2	2	2	2	2	2	2	2	0	0	2	0	0
C. glabrata	11388	2	2	2	2	2	2	2	2	1	2	2	1	0	0	0
P. variotii	11405	2	2	2	2	2	2	2	2	1	2	1	0	2	0	0
C. tropicalis	11392	2	2	2	2	2	2	2	2	1	2	2	0	0	0	0

The number in the cells indicates the viable disks for each condition assayed where 2 means 2/2 viable disks; 1 means 1/2 viable disks; 0 means 0/2 viable disks.

[a] Fungal Culture Collection, Mycology Department, INEI ''Dr. Carlos G. Malbrán''.

Table 4 Long-term stability

Strain	DMic number[a]	Months																	
		1	6	12	18	24	30	1	6	12	18	24	30	1	6	12	18	24	30
		−20 ± 5 °C						5 ± 3 °C						27 ± 3 °C					
A. niger	103781	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
A. tubingensis	84052	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
R. arrhizus	124205	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	0
A. fumigatus	093603	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	0	0
C. gattii	073122	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	0	0
C. neoformans	083456	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	0	0
A. flavus	113984	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	0	0
A. terreus	134330	2	2	2	2	2	2	2	2	2	2	2	2	2	2	0	0	0	0
P. variotii	114056	2	2	2	2	2	2	2	2	2	2	2	2	2	0	0	0	0	0
C. krusei	134341	2	2	2	2	2	2	2	2	2	2	2	0	2	0	0	0	0	0
C. parapsilosis	113911	2	2	2	2	2	2	2	2	2	2	2	0	2	0	0	0	0	0
C. albicans	113874	2	2	2	2	2	2	2	2	2	0	0	0	2	0	0	0	0	0
C. tropicalis	113920	2	2	2	2	2	2	2	2	2	0	0	0	0	0	0	0	0	0
C. glabrata	113884	2	2	2	2	2	2	2	2	2	0	0	0	0	0	0	0	0	0

The number in the cells indicates the viable disks for each condition assayed where 2 means 2/2 viable disks; 1 means 1/2 viable disks; 0 means 0/2 viable disks.

[a] Fungal Culture Collection, Mycology Department, INEI ''Dr. Carlos G. Malbrán''.

genotypic identification results were concordant and thus, were considered reference cultures.

Reference microbial culture disks of *C. krusei* and *C. neoformans* were received back from laboratories participating in the PNCCM. All laboratories surveyed showed disks in good conditions, pure and viable. Strain identification was correct in 95% and 92% of the laboratories for *C. krusei* and *C. neoformans*, respectively. This indicates that produced RFC were stable during transportation.

Discussion and conclusions

Global society needs to apply standardized methodology in order to compare results to avoid duplicating measurements that cost time and money. The need for mutual recognition, *i.e.* the possibility of comparing results in a transparent way, explains the reason why quality standards place emphasis on traceability requirements.

Continuous technological progress in the area of microbiology leads to the implementation of laboratory quality management systems to improve traceability and confidence of results. Consequently, the demand exceeds the supply in terms of the variety of reference materials available in the market, especially in the field of microbiology. The same applies to microbiological proficiency testing programs in health, cosmetics, food or environmental areas that require test items produced from microorganism cultures. In that sense, the use of native and regionally circulating microorganisms allows to evaluate field laboratories through items that are similar and comparable with routine samples.

Local RFC are essential for quality assurance and are recommended for external and internal quality control programs. Moreover, they are of utmost importance when generated results have an impact on population health. Furthermore, the use of regional cultures prevents the entry of foreign strains that could damage natural flora development or generate disease foci in humans, plants and animals affecting the regional ecology.

In this work, traceability of the strains used for the production of these cultures allows independence in the use of microorganisms from a given collection, since the identification of the microorganism is defined at the genus and species levels and the characterization by two different reference methods allowed us to confirm the identity of the strains and their equivalence with others from international collections.

Preparation of an initial master cell bank preserved both in liquid nitrogen and at $-70 \pm 10\,^{\circ}C$ allows the use of the same seed lot in different production batches, ensuring the stability and traceability of the RFC since microorganisms are metabolically inactive under these storage conditions. Cryopreservation also ensures excellent long-term viability, absence of genotypic changes and maintenance of the properties of RFC. On the other hand, ultra-cold storage at $-70 \pm 10\,^{\circ}C$ proved to be adequate and convenient for the maintenance of stock batches (which are most frequently used to start the production process). Although cultures remain viable for a shorter period of time than in liquid nitrogen and could have metabolic changes, ultra-cold storage is considered a very efficient conservation method in the medium term. Maintenance cost is lower, fewer infrastructure is required, is easy-to-use and does not require continuous supply or special rooms necessary to ensure the safety of the personnel that handle cultures stored in liquid nitrogen tanks. In fact, these two methods are commonly used by Culture Collections of Microorganisms in order to preserve biodiversity of species, including those that are used in biotechnological processes[1].

Desiccation on filter paper was chosen as the conservation method in the medium term for the production of the distribution batches. The shelf life of RFC disks with mycelial fungi is at least two years when stored in a refrigerator or at $-20 \pm 5\,^{\circ}C$. However, yeast RC disks showed a decrease in viability after one year at $5 \pm 3\,^{\circ}C$; therefore, we recommend that they should be stored at $-20 \pm 5\,^{\circ}C$ for up to two years. Both conditions are usually available in clinical microbiology laboratories minimizing the resources required for storage.

Using paper disks as support media allowed distribution of three identical replicas in one vial. They remained pure and stable for at least 7 days at $27 \pm 3\,^{\circ}C$, maximum estimated time for transportation without refrigeration. These results were supported by those obtained for yeast RC using the PNCCM.

Undoubtedly, production and quality control processes established by our work group following the ISO Guide 34:2009 and the IRAM 14950:2010 standard, the current documents available when the production started, allowed to prepare traceable and certifiable regional microbial culture lots of proven quality and low cost[2,6].

RFCs can be used for internal quality control and the production of test items for proficiency testing programs in different fields of microbiology.

The procedures developed and documented here serve as a basis and guide for the production of RFCs of other microbial species.

Conflict of interest

The authors declare no conflict of interest.

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