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Comparison of bacterial strain preservation methods in a diagnostic laboratory

Comparación de métodos de conservación de cepas bacterianas en un laboratorio de diagnóstico

RAMOS-GONZALEZ, Elsy Janeth†*´, REYES-ESCOBEDO, Fuensanta del Rocío´´, ORTA-MARTINEZ, Felipe´´ and MARTÍNEZ-ORTIZ, Rosa María´´´

´ *Universidad Autónoma de Zacatecas, Unidad Académica de Preparatoria. México.*
´´ *Universidad Autónoma de Zacatecas, Área de Ciencias de la Salud, Unidad Académica de Ciencias Químicas. México*.
´´´*Universidad Autónoma de Zacatecas, Área de Ciencias de la Salud, Unidad Académica de Odontología. México.*

ID 1st Author: *Elsy Janeth, Ramos-Gonzalez* / **ORC ID:** 0000-0002-0572-3211

ID 1st Co-author: *Fuensanta del Rocío, Reyes-Escobedo* / **ORC ID**: 0000-0002-4052-746X

ID 2nd Co-author: *Felipe, Orta-Martinez* / **ORC ID:** 0009-0007-4118-7106

ID 3rd Co-author: *Rosa María, Martínez-Ortiz* / **ORC ID:** 0000-0001-7811-169X

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Abstract

The use of microorganisms has been key in solving health problems, in the search for energy sources, as well as in environmental conservation, among others. The study of microorganisms involves the use of live cultures, which must be maintained and conserved. This need has driven the search for methodologies that are useful for the preservation of strains that are of interest. The objective of this work was to compare three different methods of conservation and storage of strains. The results show that the periodic reseeding method is only viable to be used during strain evaluations, the mineral oil method preserves the strains well but is not viable for long periods of time. The freezing method demonstrated that the strains are preserved without alterations for long periods of time. In conclusion, the 3 conservation methods guarantee the survival of the strains, the choice of which will depend on the frequency of use of the cultures.

Bacterial cultures, Cryopreservation, Mineral oil, Periodic reseeding

Resumen

El uso de los microorganismos ha sido clave en la solución de problemas de salud, en la búsqueda de fuentes de energía, así como en la conservación del medio ambiente, entre otros. El estudio de los microorganismos involucra el uso de cultivos vivos, los cuales deberán ser mantenidos y conservados, esta necesidad ha impulsado la búsqueda de metodologías que sean útiles para la preservación de cepas que sean de interés. El objetivo de este trabajo fue comparar tres métodos diferentes de conservación y almacenamiento de cepas. Los resultados muestran que el método de resiembra periódica solo es viable para ser utilizado durante las evaluaciones de las cepas, el método de aceite mineral conserva las cepas bien pero no resulta viable para largos periodos de tiempo. El metodo de congelación demostró que las cepas se conservan sin alteraciones durante largos periodos de tiempo. En conclusión, los 3 métodos de conservación garantizan la supervivencia de las cepas, la elección del mismo dependerá de la frecuencia del uso de los cultivos.

Cultivos bacterianos, Crioconservación, Aceite mineral, Resiembra periódica

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* Author's correspondence: (E-mail: elsy.jrg@gmail.com)
† Researcher contributing as first author.

Introduction

Microorganisms have an enormous impact on life and on the physical and chemical composition of our planet (Jawetz et.al., 2016). The use of microorganisms has been key in solving serious problems for humanity, in animal and human health, in the search for new sources of energy, as well as in the conservation of the environment. The study of microorganisms involves the use of live cultures, which must be maintained and preserved in a Microbial Culture Collection to guarantee their future availability (Montes de Oca N., et.al. 2008).

Microbial collections or strains are genetic resource centres that preserve microorganisms, guaranteeing the availability of biological material for teaching and research activities (Gutiérrez-Jiménez J., et.al. 2015). Likewise, a collection of microbial cultures must maintain the strains in a viable state, without morphological, biochemical, physiological or genetic changes (Montes de Oca N., et.al. 2008). The preservation of microbial strains depends entirely on a good preservation method, which must guarantee the survival of at least 70% of the cells for a considerable period of time, so that the surviving population resembles the original, retains the important properties of the cultures and minimises the occurrence of genetic events. It should also minimise the risk of contamination and allow the purity of the crop to remain unchanged (Aleman Z et.al., 2005; Cruz Aguilar M, 2020). The aim of this work was to compare three different methods of strain preservation and storage.

Methodology

A descriptive and prospective study was conducted from 16 May to 26 August 2022 in the Cepario laboratory of the Laboratorio Estatal de Salud Pública (LESP) of the state of Zacatecas. Strains with American Type Collection Culture (ATCC) characteristics received on AMIES transport media and blood agar base (BAB) were recovered and identified. Subsequently, three methods of preservation of these strains were carried out in order to use them as reference material.

The study population included pure strains with previously typed ATCC characteristics. Strains without ATCC characteristics and strains that did not meet the sample receipt criteria were excluded. The strains included were the following: *Escherichia coli* ATCC 25922; *Klebsiella pneumoniae* ATCC 13883; *Salmonella typhimurium* ATCC14028; *Salmonella enteritidis* ATCC 49222; *Shigella boydii* ATCC 12027; *Shigella sonnei* ATCC 9290; *Shigella flexneri* ATCC 12022; *Vibrio parahaemolyticcus* ATCC 43996; *Bordetella pertussis* ATCC8467; *Bordetella parapertussis* ATCC 15311; *Bordetella bronchiseptica* ATCC 10580; *Haemophilus influenzae* ATCC10211; *Haemophilus parainfluenzae* ATCC7901; *Moraxella catarrhalis* ATCC 49143; *Staphylococcus aureus* ATCC 25923; *Staphylococcus epidermidis* ATCC 12228; *Streptococcus agalactiae* ATCC12386; *Streptococcus pneumoniae* ATCC 49619; *Enterococcus faecalis* ATCC 29212; *Neisseria polysaccharea* ATCC 43768.

The following preservation tests were performed: 1) The periodic reseeding method consists of short-term preservation in AMIES transport medium and in BAB transport medium. Briefly, a batch of a pure culture of 24 hours incubation is taken, mass inoculated onto a plate of suitable culture medium depending on the micro-organism, incubated at 36 ± 2°C for 24 to 72 hours in partial tension of 5-10% CO2 for anaerobic bacteria and 18 to 24 hours at 36 ± 2°C for aerobic bacteria; after the required time the entire bacterial growth is harvested with a sterile rayon or dacron swab and deposited in a culture tube with AMIES transport medium, while for the tube with BAB transport medium the bacterial growth is deposited in the flute spout with a loop. The tube is then sealed with Parafilm and placed in a rack at room temperature. Reseeding was carried out once a week, or as often as necessary depending on the number of tests performed. 2) Preservation with mineral oil. A pure strain in BAB transport medium is required. Briefly, with a 0.01 mm loop, a small amount of bacterial growth is taken from the selective medium in a tube of BAB. Subsequently, streak the flute beak and the bottom of the agar. Incubate for 24 hours at a temperature of 35 ± 2°C. After this time, sterile mineral oil is added up to 1 mm beyond the flute tip.

ISSN 2524-2075

Finally, the tube stopper is sealed with Parafilm and placed in a rack at room temperature. 3) Preservation by freezing at a temperature of -80°C. Briefly, from the pure culture on blood agar or tryptine soy agar, a roast of the strain is selected and inoculated into the previously identified cryotube containing 1 ml of Brain Heart Infusion (BHI) broth + 30% glycerol + 10% Milk-Light with the strain to be preserved. Parafilm was then placed around the cryotube plug. Decontaminate with 70% ethanol gauze and incubate at 2-8°C for 30 minutes. After this time, they are transferred to -80°C until use. To recover the strains that were at -80°C, the following steps were taken. The strain was thawed by placing it for half an hour at 2 - 8°C for 30 minutes in broth, until completely thawed. The vial was cleaned with a gauze soaked in 70% ethanol, then opened and the mouth of the vial was flamed and held vertically. Subsequently, the cryotube was opened and a roast was taken aseptically with a 0.01-gauge loop and reseeded on a selective medium depending on the strain in question. Finally, it is incubated at 36 ± 2°C for 18 to 24 hours or 48 to 72 hours depending on the micro-organism.

For the verification of viability and purity of the strain on the selective agar, morphological characteristics, size, colour, consistency, biochemical tests were analysed and compared with those reported by reference centres.

Results

Strain recovery from AMIES or BAB.

For the recovery of strains *Escherichia coli, Klebsiella pneumoniae, Shigella boydii, Shigella sonnei, Shigella flexneri, Bordetella pertussis, Bordetella parapertussis, Bordetella bronchiseptica, Haemophilus influenzae, Haemophilus parainfluenzae, Moraxella catarrhalis, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus agalactiae, Streptococcus pneumoniae, Enterococcus faecalis, Neisseria polysaccharea* BHI broth was used as the liquid medium; for *Salmonella typhimurium* y *Salmonella enteritidis* Tetrationane and Rapapport broths were specifically used; for the strain from *Vibrio parahaemolyticcus* 1% APA (alkaline peptonised water) was used as liquid enrichment medium.

The strains that were recovered on solid media, such as blood agar, chocolate agar, Thayer-Martin agar and charcoal agar, were the *Haemophilus spp, Neisseria spp, Haemophilus influenzae* and for *Bordetella spp*. For the rest of the strains, only trypticasein soy agar (AST) was used.

Good colony development was observed for each of the strains recovered under the conditions described above, particularly requiring the presence of oxygen. *Escherichia coli, Klebsiella pneumoniae, Shigella boydii, Shigella sonnei, Shigella flexneri, Salmonella typhimurium y Salmonella enteritidis, Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis*; for 24 to 48 hours with aerobiosis the *Vibrio parahaemolyticcus*, and 48 to 72 hours of incubation with partial pressure CO_2 *Streptococcus agalactiae, Streptococcus pneumoniae, Haemophilus influenzae, Haemophilus parainfluenzae, Neisseria polysaccharea, Moraxella catarrhalis*; strains with aerobic incubation in a plastic bag to avoid drying out of the culture medium *Bordetella pertussis, Bordetella parapertussis, Bordetella bronchiseptica*.

Macroscopic observation of the recovered strains

In AST, the strains showed the following characteristics. *Escherichia coli* had medium-sized circular colonies of translucent white colour, convex elevation and shiny surface; *Klebsiella pneumoniae* 3 to 4mm colonies with regular edges, shiny with a mucoid consistency; *Salmonella typhimurium* pale yellow round colonies, 2 to 3 mm in diameter, convex and smooth; *Salmonella enteritidis* colonies 1 to 2 mm in diameter, circular, smooth, convex, colourless and convex; *Shigella boydii* presented circular, transparent colonies with distinct borders, small; *Shigella sonnei* transparent, translucent or opaque colonies, with well-defined circular shape; *Shigella flexneri* presented transparent, translucent or opaque colonies and are usually smooth, well-defined borders; *Vibrio parahaemolyticcus* small, circular yellow colonies with well-defined edges; *Staphylococcus aureus* cream to light yellow pigmented colonies, 1 to 3 mm in diameter, smooth, slightly raised with entire margins, slightly convex; *Streptococcus agalactiae* grew as very small transparent or opaque colonies; *Staphylococcus epidermidis*

ISSN 2524-2075

presented light yellow colonies, which are in the form of grape clusters (figure 1).

On charcoal agar, the strains showed the following characteristics: *Bordetella pertussis* had colonies 1 mm in diameter with the appearance of greyish mercury droplets, small; *Bordetella parapertussis* had greyish-coloured colonies 1 mm in diameter; *Bordetella bronchiseptica* presented smooth convex shiny convex almost transparent grey coloured colonies; *Haemophilus influenzae* had opaque, smooth, greyish, pearl-like, opaque colonies, 1mm in diameter (figure 1).

On blood agar *Staphylococcus epidermidis* presented greyish colonies, 1mm in diameter, with alpha haemolysis; *Streptococcus agalactiae* presented small greyish shiny colonies, with Beta haemolysis; *Enterococcus faecalis* presented very small, opaque, light grey colonies 0.5 to 1 mm in diameter, with alpha haemolysis (figure 1).

On chocolate agar *Streptococcus pneumoniae* presented small whitish-white transparent colonies depressed in the centre, presented alpha haemolysis, convex and mucosal; *Moraxella catarrhalis* presented small colonies 1mm in diameter with a clear halo, round, smooth, shiny surfaces, and well-defined borders (figure 1).

Biochemical tests

The results of the biochemical tests based on the metabolism of sugars, specific amino acids such as lysine, citrate, for the presence/absence of urease of the strains included have the same result as reported by the Institute of Diagnosis and Epidemiological Reference (INDRE), so our strains have the metabolic characteristics that identify each of the strains.

Only the strains *Moraxella catarrhalis, Bordetella bronchiseptica* y *Bordetella pertussis* strains presented the intracellular oxidase enzyme; the strains *Moraxella catarrhalis, Staphhylococcus aureus, Bordetella parapertussis* and *Staphylococcus epidermidis* demonstrated the presence of the enzyme catalase.

Preservation methods

Periodic reseeding method. All strains that were transferred at room temperature to the BAB transport medium, until their fourth pass, allowed survival of the cultures in short periods of time of 15 to 20 days, a high risk of contamination was observed. In addition to the fact that there may be variability in the characteristics presented by each of the strains, due to the constant passages to which the strains are subjected.

Preservation with mineral oil. The strains that were preserved by the mineral oil technique were *Escherichia coli, Klebsiella pneumoniae, Salmonella typhimurium, Salmonella diarizonae, Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis, Shigella sonnei, Shigella flexneri, Shigella boydii* y *Vibrio parahaemolyticcus*.

Preservation by freezing at -80°C. The strains were kept frozen from June until their recovery in August. All strains that were selected for recovery grew on all selected seed media at a temperature and incubation time required for each strain. Particularly *Bordetella pertusis* y *Bordetella bronchiseptica* did not show growth after 48 hours of incubation on any of the selected media. However, at 72 hours *Bordetella parapertussis* and *Bordetella bronchiseptica* showed growth on all selected media. *Haemophilus influenzae* growth on chocolate and charcoal agar at 48 hours, while at 72 hours, colonies were present on all selected media. *Streptococcus pneumoniae* grows on all selected media after 48 hours of incubation.

Discussion

The main purpose of the collection of microorganisms within a diagnostic or research laboratory is the preservation of the viability of each of the bacterial strains used as reference material (Montes de Oca N., et.al. 2008). The LESP has a Microbial Culture Collection with reference strains obtained from a collection such as the American Type Collection Culture (ATCC). These strains are preserved in three preservation methods, a short-term one: periodic reseeding, a medium-term one: the mineral oil method, and finally a long-term one: the -80°C freezing method.

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Periodic reseeding is a form of safeguarding. The micro-organism in question is seeded in its appropriate medium and once grown, it is stored at 4°C where it is kept for a few days for use and reseeded in no more than a month. The big problem with the continuous reseeding of a microorganism is that it can generate mutations and adaptation to the culture medium, which ends up generating "domesticated" strains whose behaviour no longer represents the initially isolated species (Morales-García YE, et al. 2010). Periodic reseeding results in fresh cultures that can be used as working material in each of the areas as required. Once the fourth pass has been reached, a crop must be obtained from the mother crop. This can be considered a disadvantage for use as reference material for epidemiological diagnostics or other future evaluations.

For medium-term preservation, the mineral oil technique was used. This method covers a culture grown in a liquid or solid medium with a thin layer of 1 to 2 cm with a sterile, non-toxic mineral oil, most commonly paraffin or petroleum jelly. The aim is to limit the access of oxygen to the culture, which will reduce the metabolism and growth of micro-organisms (Uzunova-Doneva T, 2004-2005). These characteristics mean that mineral oil can preserve the strains for a longer period of time, thus avoiding the excessive use of passages that could promote phenotypic or genotypic changes. An advantage of using mineral oil is that it avoids desiccation of the culture medium, which could be toxic for the microorganisms, as well as being a low-cost technique.

Long-term preservation involves storing cells at very low temperatures. Under these conditions water, the major component of living cells, changes from a liquid to a solid phase. Lower temperatures of -20 to -70°C favour viability and genetic stability for a period of more than thirty years. There are many factors that can affect the viability and stability of cultures during the freezing process, such as the age of the cells, the freeze-thaw rate, the storage temperature and the use of cryoprotective agents. Among the most commonly used cryoprotectants are glycerol and skimmed milk (Gato Cárdenas Y. 2010). This technique forces the bacteria to adapt to the new adverse conditions of the environment they are being provided with, which causes a change in the speed of their metabolism.

This preserves their viability and prevents irreversible damage to the specific characteristics of each bacterium. The advantage of this technique is that when the conditions necessary for their proliferation return, the bacteria recover their metabolism, returning to obtain a live culture with the original phenotypic and genotypic characteristics. Furthermore, this technique can be applied to most microbial genera, reducing the risk of contamination and allowing the microorganisms to be maintained in the long term (García MD 2001; Gato Cárdenas Y. 2010; Morales-García YE, et al. 2010). One of the disadvantages of this technique is that it requires the implementation of equipment that is very expensive and not all laboratories have access to it. In addition, continuous freezing and thawing processes can cause changes in the cells.

Conclusion

The results obtained show that the 3 methods of preservation (by periodic reseeding, by mineral oil and by freezing at -80°C), guarantee the survival of the strains for a period of time determined by the method used. In order to choose the appropriate method, the frequency of use of the cultures must be taken into account.

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