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Preservation Evaluation of *Mycoplasma fermentans* in Different Conditions

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Abstract: Different species of mycoplasmas are considered pathogenic in humans, in addition to being characterized by their difficult cultivation in the laboratory and demanding nutritional requirements, their preservation is complicated for future research based on clinical isolates. The objective of this work was to evaluate the viability of *Mycoplasma fermentans* preserving it with different cryopreservatives and temperatures. The preservation evaluation of *Mycoplasma fermentans* was performed with SP4 broth supplemented with 40% fetal bovine serum (FBS) and 10% dimethylsulfoxide (DMSO), 40% gelatin (GEL) and 10% DMSO, 40% glycerol (GLI) and 10% DMSO, and supplement free SP4 broth as a control. 10 mL of each of the SP4 broths supplemented with 10 mL of *Mycoplasma fermentans* culture were mixed at a concentration of $1X10^6$ CFU/mL, stored at -20°C and -80°C for twelve months. Every two months, it was thawed and its viability was evaluated on SP4 agar and quantification of the CFU/mL. Trials with fetal bovine serum, gelatin and glycerol at -20°C and -80°C showed efficiency in keeping *Mycoplasma fermentans* viable during the twelve months. Glycerol being the cryopreservative agent that favored the greatest viability, showing significant difference ($P < 0.05$), with respect to the control group. Supplementation of SP4 broth with glycerol showed the best viability for *Mycoplasma fermentans*.

Keywords: Preservation, *Mycoplasma fermentans*, Fetal Bovine Serum, Glycerol, Dimethylsulfoxide

Introduction

Mycoplasmas are bacteria that lack a cell wall, have demanding nutritional requirements and are characterized by frequently contaminating cell cultures, causing diseases in plants, animals and humans (1,2). *Mycoplasma fermentans* colonizes humans and is considered potentially pathogenic in rheumatic, genital, respiratory diseases and oncogenic properties have been described, in addition to acting as a cofactor in the progression of HIV (3-7). However, the mycoplasma-host cell interaction is not yet fully studied, due to the difficulty of the culture and its preservation.

It has been reported that variations in conservation temperature and that the freezing and thawing process induce changes in the growth and size of mycoplasma colonies (8). An investigation evaluated the viability of mycoplasma cultures of animal origin during periods of two years at different freezing temperatures and in the lyophilized phase, finding that their viability was favored, but involved more infrastructure and high operating costs, highlighting that there are various reports that study the protocols to maintain the viability of mycoplasmas, the frequently described species being of veterinary interest (9-12).

Few reports involve protocols to maintain the viability of mycoplasmas of human origin and interest in public health, where cold conservation protocols stand out for periods of six to ten years, but diminishing their viability (13). The cold prolongs the biological time since it slows down the cellular reactions; However, this is not a problem-free process since it can induce changes in the chemical, thermal and electrical properties which can alter the cell membranes of the samples to be preserved. Thus, the preservation and storage of biological material is of importance, especially for mycoplasmas of human origin, since the few conservation methods reported are not completely effective. Therefore, the objective of the present work was to evaluate the viability of *Mycoplasma fermentans* keeping it with different cryopreservatives and temperatures.

Material and methods

Mycoplasma fermentans P140 provided by the mycoplasma laboratory of the Research Center in

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Microbiological Sciences, Institute of Sciences of the Benemerita Autonomous University of Puebla, was used, which was isolated from the airways of an asthmatic patient, and It was characterized by microbiological culture and polymerase chain reaction (PCR) with oligonucleotides AR1-AR2 and RW004-RW005 to determine gender and species, and as a strain of reference was used *Mycoplasma fermentans* PG18 (ATCC® 19989 ™) (14-16).

Both mycoplasmas were grown separately in SP4 broth (containing for a volume of 100 mL: 2 grams of mycoplasma base, 0.5 grams of dextrose, 1 mL of 0.4% phenol red, 65 mL of distilled water, 10 mL of 25% yeast dialysate and 25 mL of horse serum) and its viability was confirmed in SP4 agar (containing for a volume of 100 mL: 2 grams of mycoplasma base, 0.5 grams of dextrose, 1.5 grams of agar, 65 mL of distilled water, 10 mL of 25% yeast dialysate and 25 mL of horse serum), preparing an inoculum at a concentration of $1X10^6$ CFU / mL, for *Mycoplasma fermentans* P140 and PG18, respectively.

The preservation evaluation of *Mycoplasma fermentans* P140 and PG18 was carried out with the following tests: (a) 100 mL of SP4 broth supplemented with 40% fetal bovine serum (FBS) and 10% dimethylsulfoxide (DMSO), (b) 100 mL of SP4 broth supplemented with 40% gelatin (GEL) and 10% DMSO, (c) 100 mL of SP4 broth supplemented with 40% glycerol (GLY) and 10% DMSO and (d) 100 mL of SP4 broth supplement free as a control. 10 mL of each of the SP4 broths supplemented with 10 mL of *Mycoplasma fermentans* P140 culture were mixed at a concentration of $1X10^6$ CFU/mL, with twelve replicates each. From each trial, six replicas were stored at -20°C and the remaining six at -80°C for twelve months. A sample of the different trials was thawed every two months and its viability was evaluated by seeding on SP4 agar and quantification of the CFU/mL by stereoscopic microscopy (Nikon SMZ-2T Japan). For *Mycoplasma fermentans* PG18 the same working scheme was applied. To evaluate the difference in the viability of mycoplasmas in the temperatures and cryopreservants tested, an analysis of variance was performed with a significance level of 0.05 (IBM SPSS Statistics Software).

Results and discussion

The use of microorganisms has been important in medical research, for which reason its study requires its conservation and storage to guarantee the availability of microbial cultures, especially mycoplasmas that have demanding nutritional requirements, because traditional conservation methods do not They are fully compatible with these bacteria (17). The use of low temperatures affects diffusion and osmosis across membranes, since each cell type has its own biophysical profile which interacts with different cellular cryopreservatives. For this reason, knowing the proper preservation protocol will guarantee the viability and functionality of mycoplasmas of medical interest (18).

The tests carried out with *Mycoplasma fermentans* P140 and PG18 in the presence of fetal bovine serum, gelatin and glycerol at -20°C and -80°C showed efficiency to keep the cultures viable at twelve months preserved (Figure 1). Glycerol being the cryopreservative agent that favored the greatest viability at both freezing temperatures, obtaining the highest viability at -80°C in *Mycoplasma fermentans* P140 with 78% and *Mycoplasma fermentans* PG18 with 83% (Figure 1 panel B and D), showing significant difference ($P < 0.05$) with respect to the control group. The results of viability for *Mycoplasma fermentans* P140 and PG18 in the presence of fetal bovine serum and glycerol at -20°C and -80°C were remarkable and are consistent with that reported for other species, as documented in different cryopreservation protocols where combination of glycerol with dimethylsulfoxide improved the viability and integrity of post-defrosting membranes, and in preservation trials of fibroblast cultures in the presence of fetal bovine serum where an improvement in cell survival, development and proliferation capacity was obtained (19,20).

The variations obtained in the viability between *Mycoplasma fermentans* P140 and PG18 are related to the interaction that occurs when supplementing the SP4 broth with the different cryopreservatives, similar to that reported in a study involving *Mycoplasma bovis*, *Mycoplasma californicum*, *Mycoplasma canadense*, *Mycoplasma bovigenitalium* and *Mycoplasma alkalescens* where the times, temperatures and the addition of glycerol were evaluated for preservation, determining that the supplementation of milk with glycerol at -20°C for five days favored the viability and the re-isolation of the species evaluated (21).

With respect to the two temperature ranges that were used, there was no marked variation, favoring the viability of *Mycoplasma fermentans* P140 and PG18 during the twelve months of the test, evidencing by means of the culture in SP4 agar with a characteristic colonial growth of mycoplasmas with considerable diameters and abundant growth, as was the case with *Mycoplasma fermentans* P140 (Figure 2 panel A and B) and *Mycoplasma fermentans* PG18 (Figure 2 panel D).

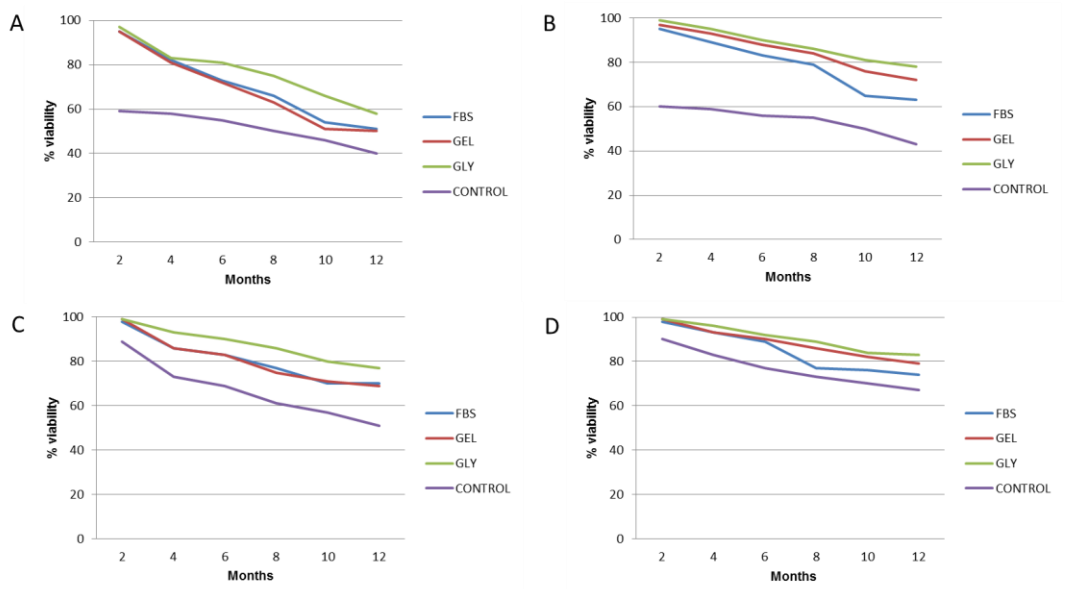

Figure 1. *Mycoplasma fermentans* P140 viability for twelve months (A) -20°C and (B) -80°C and *Mycoplasma fermentans* PG18 (C) -20°C and (D) -80°C. FBS = fetal bovine serum, GEL = gelatin, GLY = glycerol.

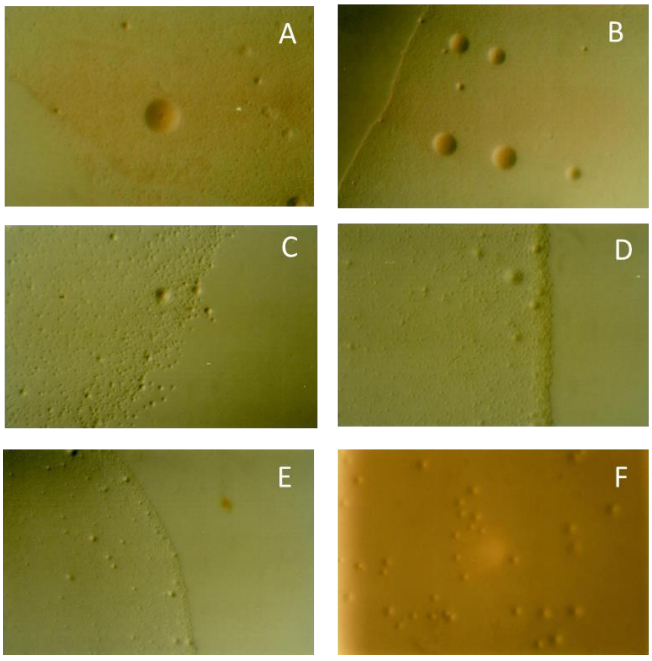

Figure 2. Colonial growth characteristic of mycoplasmas, the viability *Mycoplasma fermentans* P140 is presented at month twelve with preservation in glycerol (A) -20°C and (B) -80°C, *Mycoplasma fermentans* PG18 (C) -20°C and (D) -80°C and in the absence of 12 month supplements at -80°C *Mycoplasma fermentans* P140 (E) and PG18 (F), stereoscopic microscopy (4X).

The viability obtained with *Mycoplasma fermentans* P140 and PG18 under preservation conditions corresponds to the evaluation of the viability of *Mycoplasma hyorhinis* in the presence of glycerol and dimethylsulfoxide cryopreservative agents at -20°C and -70°C for 60 and 90 days, showing that This mycoplasma remains viable at 90 days in both temperature ranges with respect to the control. It is even suggested that at -20°C it can be used in laboratories that lack equipment at -70°C for the preservation of samples for a period of three months (22), on the other hand the tests with *Mycoplasma bovis* in the presence of fetal bovine serum, gelatin, glycerol and dimethylsulfoxide at -4°C, -20°C and -80°C, its viability with respect to the control was favored, with the combination of glycerol and dimethyl sulfoxide which favored 57% of viability with respect to 19% of the control during week 16 at a temperature of -80°C (23).

Regarding the effect on the variation in the colonial growth of *Mycoplasma fermentans* P140 and PG18, this behavior is related to the absence of cell wall that characterizes mycoplasmas, an effect that has been reported in other work (Kelton, 1964) and given the diversity intraspecific of the microorganisms, it has been established that not all strains of the same species respond equally to the selection of tests applied to them (24,25).

The action of the cryopreservatives used in the present work is attributed to their ability to prevent electrolyte accumulation during the freezing process and the formation of ice crystals which damage the structure of the mycoplasma membrane, as well as affect the processes of water solvation.[18] In conclusion, the SP4 broth supplemented with 40% glycerol and 10% dimethylsulfoxide showed the best results during the twelve months at -20oC and -80oC in the evaluation of the viability of *Mycoplasma*

fermentans P140 and PG18. Not forgetting that to obtain an ideal protocol to preserve mycoplasmas depends on the physicochemical properties of the microorganism, since this process is conditioned by different variables such as the species, origin and stage of the sample to be preserved.

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