

Impact of various preservation and storage methods on the viability of mycoplasma field strains isolated in Mali

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Abstract

The survival of five mycoplasma strains was studied in different storage media (mycoplasma complete media without cryopreservative agent, mycoplasma complete media with addition of horse serum, mycoplasma complete media with addition of glycerol and lyophilized cultures without stabilizer) under different temperatures (+37 °C, +4 °C, -20 °C, -85 °C) for 24 months. Five mycoplasma strains, *Mycoplasma mycoides mycoides*, *Mycoplasma bovis*, *Mycoplasma agalactiae*, *Mycoplasma gallisepticum* and *Mycoplasma synoviae* were isolated from various parts of the country. The initial titers of the strains determined by the agar plate count before storage were 42.4x10⁷ UFC/ml (8.6 log UFC/ml) for *Mycoplasma mycoides mycoides* strain; 32.4x10⁸ UFC/ml (9.51 log UFC/ml) for *Mycoplasma bovis* strain; 12.4x10⁹ UFC/ml (10.09 log UFC/ml) for *Mycoplasma agalactiae* strains; 2.4x10⁹ UFC/ml (9.38 log UFC/ml) for *Mycoplasma gallisepticum* and 2.8x10⁹ UFC/ml (9.45 log UFC/ml) for *Mycoplasma synoviae* strain. After 3 weeks of storage, no viable mycoplasmas were detected in all the conservation media at +37 °C and after 3 months of storage at +4 °C except for the lyophilized cultures in which an average viability rate of 17.81 % was observed. Overall, the mycoplasma strains remained viable at freezing temperatures after 24 months regardless of the storage medium, but with decreasing titers, which was noticeable with mycoplasma complete media, and mycoplasma media with horse serum. Conversely, at -20 °C the average viability rates after 24 months of storage were 84.36 % (with glycerol) and 90.04 % (lyophilized cultures). At -85 °C after 24 months of storage, this was 87.98 % (with glycerol) and 91.44 % (lyophilized cultures). These findings suggest that, in the absence of the lyophilization process, the addition of glycerol may be recommended for long-term storage of frozen mycoplasma isolates.

Keywords: Conservation; Mycoplasma Strains; Culture Medium; Horse Serum; Glycerol

1. Introduction

Mycoplasmas are bacteria that lack a cell wall, have demanding nutritional requirements and are characterized by causing diseases in plants, animals and humans and frequently contaminate cell cultures. The absence of a rigid cell wall makes them potentially vulnerable to environmental changes, but they can nevertheless be preserved by low temperature storage, especially by freezing. Numerous studies have been carried out on the epidemiology and control of diseases caused by mycoplasmas by isolating the wild strains responsible for pneumonia, mastitis and arthritis [1–

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6]. However, very few studies have been conducted on methods of storing specimens and isolates of mycoplasmas using storage media and temperatures. The duration of the viability of mycoplasma strains under storage conditions depends on several factors, including the nature of the culture medium, as the protein content is probably responsible for the stability of the mycoplasmas over a long period [7] and also on temperature as well as addition of cryopreservatives agents such as glycerol and dimethyl sulfoxide (DMSO). During the storage of milk samples, the shelf life of *Mycoplasma agalactiae* and *Mycoplasma mycoides subsp. capri* was dependent on the temperature of refrigeration and freezing [8]. Cryopreservative agents are thought to improve the survival of *Mycoplasma bovis* in milk for both transport and long-term storage [9]. The critical effect of the process of thawing was evaluated with milk samples for the detection of mycoplasmas where thawing at room temperature was better than in a water bath at +37 °C [10]. Similarly, with respect to the effect of the environment, strains of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* remained viable for up to 2 to 4 days and 2 to 3 days, respectively [11]. Thus, appropriate storage conditions are necessary to achieve maximum survival of the mycoplasma strains. A cooling rate of 1-3 °C/min during the initial freezing phase (+4 °C to -40 °C) is optimal for most mammalian cells when frozen in the presence of cryoprotective agents, such as glycerol or DMSO [12]. The most important variables are the rates of cooling and warming, and the type and concentration of cryoprotectant. In general, as the concentration of cryoprotectant is increased, cell survival improves [13].

This study evaluated the viability of *Mycoplasma mycoides mycoides* (Mmm), *Mycoplasma bovis* (Mb), *Mycoplasma agalactiae* (Ma), *Mycoplasma gallisepticum* (Mg), *Mycoplasma synoviae* (Ms) using various storage media (Hayflick complete medium without cryopreservative agent, Hayflick with addition of horse serum and Hayflick with addition of glycerol) and lyophilized cultures of each strain after 24 months of storage under four different storage temperature ranges (+37 °C, +4 °C, -20 °C and -85 °C).

2. Material and method

2.1. Growth media and isolation of mycoplasma strains

Five strains of mycoplasma were obtained by isolation from lung tissue samples, pleural fluid, milk and inner ear swabs from cattle and small ruminants. Lung tissue samples were obtained in abattoirs after post-mortem inspection. Routinely, the bleeding of animals in all abattoirs is carried out after mechanical stunning with a perforating rod gun (or matador). These were strains of Mmm, responsible for contagious bovine pleuropneumonia, Mb, responsible for pneumopathies and mastitis in cattle, Ma for contagious agalactia in young ruminants, Mg and Ms, responsible for avian mycoplasmosis. These organisms were cultured in three specific media, which were Gourley's medium (Bacto tryptose, Glucose, Sodium chloride, Sodium Phosphate Dissodique, Glycerol, Yeast Extract) [14] for the isolation of Mmm, the modified Hayflick medium [15] (Glucose, Sodium pyruvate) for Mb and Ma and Frey medium (Glucose, NAD, Thallium acetate) [16] for Mg and Ms. The base medium (PPL0 broth, PPL0 agar) was prepared in the proportion of 42 g of PPL0 and 10 g of noble agar / liter then sterilized by autoclaving at 121 °C for 15 min. As for the mycoplasma growth supplements composed of horse serum, fresh yeast extract, thallium acetate and ampicillin, they were sterilized by filtration through 0.45 µm then 0.22 µm. The complete mycoplasma medium was composed of basal medium (70 %) and mycoplasma growth supplements (30 %) (i.e. 70 ml of PPL0 and 30 ml of growth supplement).

2.2. *Mycoplasma mycoides mycoides* (Mmm) growth medium

Gourley's medium for *Mycoplasma mycoides mycoides* isolation : Bacto tryptose (BD / Ref 211713 / Batch 5299713), Glucose (SIGMA / Ref G5767 Batch BCBC0777), Chloride sodium (Aldrich / Ref 7647-14-5 / Batch 03609JT), Dissodium Sodium Phosphate (SIGMA / Ref 59763 / Batch BCBC3154), Glycerol (SIGMA / Ref 49770 / Batch BCBB7435), Yeast Extract (Fluka / Ref 70161 / Batch 0001439171), Horse serum (Gibco / Batch 1750660), Penicillin G (SIGMA / Ref 46609 / Batch BCBW2380); Noble agar (BBL / Ref 211456 / Batch 1112313).

2.3. *Mycoplasma bovis* (Mbovis) and *Mycoplasma agalactiae* (Ma) medium

Modified Hyflick medium for culturing *Mycoplasma bovis* and *Mycoplasma agalactiae*: Glucose (SIGMA / Batch BCBC0777), Sodium pyruvate (Sigma / Batch 115K07251), Horse serum (Gibco / Batch 1750660), Fresh yeast extract (LCV-ELF / Batch 19005), Ampicillin (Sigma / Batch BCBR6229V), Thallium acetate (Alfa Aesar / Batch: G06Y019 / Ref: 11842).

2.4. *Mycoplasma gallisepticum* (Mg) and *Mycoplasma synoviae* (Ms) growth medium

Dextrose (LAB M / Batch: Q23079 / Ref: 100743), Arginine (IGN / Batch: 3253E / Ref: 100743), Fetal calf serum (Eurobio / Ref: CVFSVF00-04 / Batch 531808), Horse serum (Gibco / Batch: 1750660 / Ref: 26050-088), Fresh yeast

extract (Ref: ELF_LCV19005 / Batch: 001), NAD (Sigma / Ref: N3014 / Batch: 30K7068), Thallium acetate (Alfa Aesar / Batch: G06Y019 / Ref: 11842), Penicillin (Sigma / Ref: 46609 / Batch: BCBW2380).

2.5. Preparation of mycoplasma suspensions for storage

Five (5) strains of pathogenic mycoplasmas were selected for their storage time in different storage media and temperatures. These local mycoplasma strains were *Mycoplasma mycoides mycoides* (Mmm); *Mycoplasma bovis* (Mbovis); *Mycoplasma agalactiae* (Ma); *Mycoplasma gallisepticum* (Mg) and *Mycoplasma synoviae* (Ms).

For cultivation, dilutions to 1/10th in eight (8) tubes (10^{-1} – 10^{-8}) to 1 ml of sample in 9 ml of growth medium (broth) were carried out then 8 dishes of agar per sample were prepared (i.e. one agar dish per dilution from -1 to -8).

On each agar dish 100 µl of the dilution are deposited and spread on the surface of agar (100 µl of the dilution -1 on the agar plate labeled -1). After incubation at +37 °C in humid atmosphere at 5% CO₂, the dishes are examined every day for bacterial growth signs (change in color of the broth and the characteristic morphology of mycoplasma colonies "in egg on flat").

The base medium (PPLO broth, PPLO agar) was prepared in the proportion of 42 g of PPLO and 10 g of noble agar/liter then sterilized by autoclaving at 121 °C for 15 min. All the growth supplements composed of horse serum, fresh yeast extract, thallium acetate and ampicillin were sterilized by filtration through 0.45 µm then 0.22 µm. The complete mycoplasma medium was composed of 70 % basal medium and 30 % growth supplement (i.e. 70 ml of PPLO and 30 ml of growth supplement).

Each diluted mycoplasma strain was aliquoted into 15 Eppendorf tubes (1 ml each) on the same day of harvest. For total of 1300 tubes of 1 ml stored, 60 tubes were at +37 °C, 120 at +4 °C, 560 at -20 °C and 560 tubes at -85 °C, i.e. 260 tubes of 1 ml of each strain. At each titration, an aliquot of each storage medium was grown on selective media. At the same time, each mycoplasma strain was lyophilized in a volume of 2.5 ml / vial produced in sufficient number for each storage temperature.

2.6. Preparation of storage media

The following storage media were selected for this study: (a) Control medium without preservative additive (M1), (b) Complete medium for mycoplasmas as a preservative additive (M2), (c) Horse serum as a preservative additive (M3), (d) 60 % glycerol as a preservative additive (M4) and (e) Lyophilizate culture without stabilizer as preservative (M5). For each preparation, 500 µl of each strain were added to 500 µl of each storage medium to have 1 ml of final volume to store (50 % and 50 %, v/v).

2.7. Determination of number of viable mycoplasmas

The effect of storage methods on the viability of mycoplasma strains was assessed by series of the agar plate count before (Day 0) and during (Week 1, Week 2, Week 3, Month 1 and Month 24) storage. All strains were subjected to the same thawing procedure. To avoid the thermal shock of rapid thawing, each strain frozen at -85 °C was first returned to freezing at -20 °C for 30 min to 1 hour then at +4°C for 15 – 30 min before titration.

In this experiment, the samples were tested at week0 (initial starting titer), week 1 (W1 post storage), week2 (W2 post storage), week 3 (W3 post storage), Month 1 (M1 post storage) to Month 24 (M24 post storage) after conservation. Each strain was tested 28 times per storage temperature (+37 °C, +4 °C, -20 °C, -85 °C) i.e. $28 \times 4 = 112$ times/strain. To avoid not only heat shock but also contamination, 1 aliquot of culture was used for each titration (i.e. 1 aliquot of culture per strain per temperature and per test). We used average titer data obtained per strain of mycoplasma; these are the titer averages on 3 agar plates/strain.

The number of viable mycoplasmas was determined by the colony count method with several dilutions to 1/10 of the mycoplasma suspensions in proportion to 0.1 ml / agar plate. The dilutions giving 30 to 300 colonies were counted by means of a stereomicroscope and the results were expressed in colony forming units (CFU) per milliliter of suspension as follows:

$$\text{Log UFC/ml} = \log(N) + \log(I) + D$$

N = number of colonies counted; I= inoculum volume on agar (volume per ml); D = dilution number;
Inoculum volume on agar reported in ml = $\log\left(\frac{1000\mu\text{l}}{100\mu\text{l}}\right)$

2.8. Statistical analysis

Data was recorded and classified on the Excel spreadsheet software (Microsoft excel 10). The statistical tests used were the log means comparison (log CFU / ml) by strain, considering the fixed effect of media and storage temperatures (+37, +4, -20 and -85 °C) by analysis of variance (ANOVA) with Stata 12.0 software and the proportions comparison (viability rate) by the chi-squared test with R software (R i386 4.0.3). A significant level of 0.05 was retained for the comparison of various parameters. Repeatability analysis improved the reliability of experimental data such as those in this article where series of repetitions of the titration of mycoplasma strains under different storage conditions (temperature, medium). For 24 consecutive months, post-storage Mycoplasma titrations were performed once weekly from W0 (week 0 storage) to W3 (three weeks storage) and once monthly from Month 1 to Month 24 post-storage. Therefore, a measurement repeatability analysis was confirmed on the mean titer data of each titration series using Stata V.12 software thus allowing explanation of the variances that occur. A one-way repeated measures ANOVA was conducted to examine the effect that five different mediums had on response time.

3. Results

Overall, the mycoplasma strains remained viable at freezing temperatures after 24 months regardless of the storage medium. However, the number of viable mycoplasmas decreased slightly over time. The decrease was much smaller at storage temperature of -85 °C than at -20 °C and in most cases, viability was higher in lyophilized cultures and glycerol than in horse serum and medium only ($p = 0.03$). On the other hand, the reduction in the viability of mycoplasma strains was much greater when they are stored at +37°C where the loss was maximum after 3 weeks (97.9 %) and at +4 °C with a maximum loss after 3 months of storage except lyophilized cultures (99.3 %). With equal variance for all combinations, global results showed a statistically significant difference between the average titers of mycoplasma storage media as a function of the duration for each temperature whatever the correction factor we use: Hunyh-Feldt (H-F), Greenhouse-Geisser (G-G) and Box's conservative epsilon.

3.1. Viability of mycoplasma strains at +37 °C

The results obtained from this study showed that at +37 °C, all the strains of mycoplasmas outside the incubation period were not viable beyond 3 weeks (Figure 1).

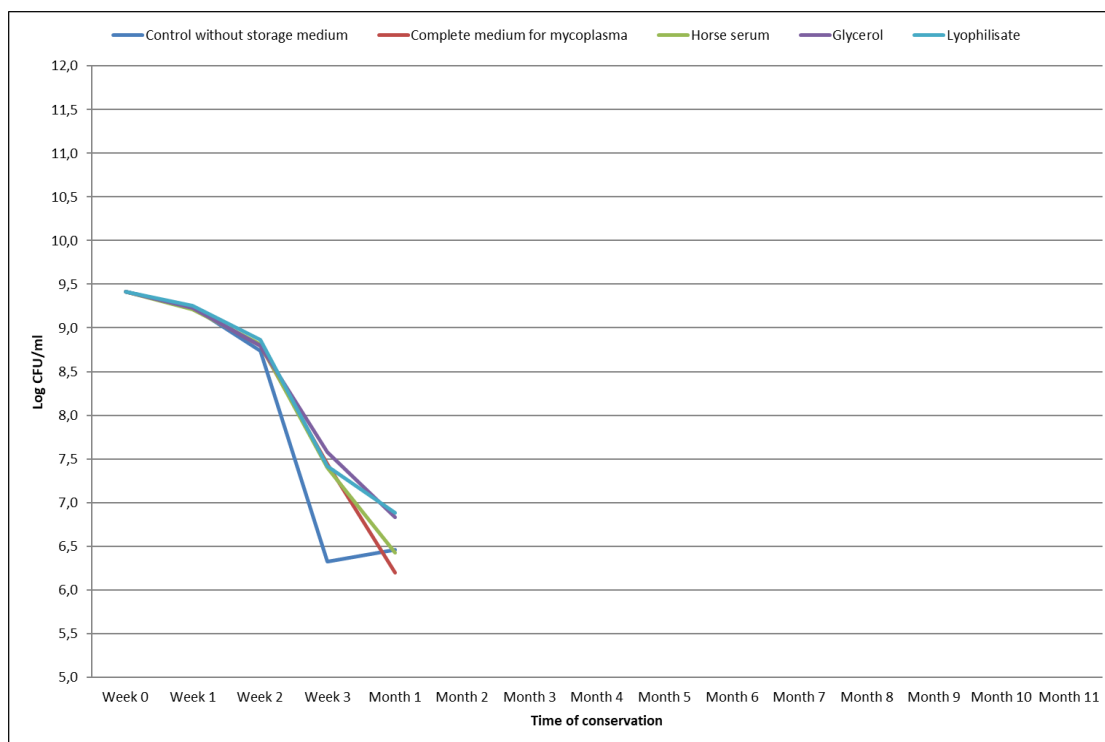


Figure 1 Mycoplasma viability at +37 °C. Mean loss (in log) of the titer of 5 strains of mycoplasmas (Mmm, Mb, Ma, Mg, Ms) during 24 months of storage in different preservatives

Overall, a very low non-significant overall mean viability ($p = 0.89$) was visible after 3 weeks of storage at +37 °C with viability rates of 2.14 %, (without preservative) at 4.38 % (lyophilized cultures). A very low viability mycoplasma culture was observed after 3 weeks of storage at +37 °C.

The viability rates varied from 5.91 % (without preservative) to 12.98 % (lyophilized cultures) for Mmm ($p = 0.42$), from 0.42 % (without preservative) at 1.16 % (lyophilized cultures) for Mb ($p = 0.95$), from 2.48 % to 5.65 %, respectively without preservative and the lyophilized cultures for Ma ($p = 0.74$), from 1.49% (without preservative) to 2.03 % (glycerol) for Mg ($p = 0.99$) and from 0.53 % (without preservative) to 0.85 % (lyophilized cultures) for Ms ($p = 0.99$).

Significant differences were observed between mycoplasma strains with addition of complete medium ($p = 0.004$), horse serum ($p = 0.0001$), glycerol ($p = 0.00026$) and with lyophilized culture ($p = 0.00072$) (Table 1).

Table 1 Viability of mycoplasma strains at + 37 °C after 3 weeks

Mycoplasma strains		Mmm ^b	Mb ^c	Ma ^d	Mg ^e	Ms ^f
Initial titer (Log CFU/ml)		8.63	9.51	10.09	9.38	9.45
Control without Preservative (M1)	Log CFU/ml ^a	0.51	0.04	0.25	0.14	0.05
	Viability rate (%)	5.91	0.42	2.48	1.49	0.53
Complete medium (M2)	Log CFU/ml	0.71	0.05	0.25	0.16	0.06
	Viability rate (%)	8.23	0.53	2.48	1.71	0.64
Horse serum (M3)	Log CFU/ml	1.03	0.10	0.41	0.17	0.07
	Viability rate (%)	11.94	1.05	4.06	1.81	0.74
Glycerol (M4)	Log CFU/ml	1.01	0.12	0.41	0.19	0.07
	Viability rate (%)	11.71	1.26	4.06	2.03	0.74
Lyophilized cultures (M5)	Log CFU/ml	1.12	0.11	0.57	0.18	0.08
	Viability rate (%)	12.98	1.16	5.65	1.92	0.85

a colony format unity per milliliter ; bMycoplasma mycoides mycoides ; cMycoplasma bovis ; dMycoplasma agalactiae ; eMycoplasma gallisepticum ; fMycoplasma synoviae

3.2. Viability of mycoplasma strains at +4 °C

The refrigeration temperature of +4 °C allowed for viable preservation of at least 3 months beyond which only the lyophilized cultures retained their viability with a loss of less than one (1) log CFU/ml (Figure 2).

At +4 °C, a significant effect ($p = 0.000417$) was observed with the storage media where the highest viability rate was obtained with the lyophilized cultures (17.81 %) and the lowest with the glycerol (2.13 %) after 6 months of storage.

The viability rates at 3 months of storage varied according to the mycoplasma strains and the storage media. A significant difference was observed with Mmm strain (0.46 % and 17.85 %, $p < 0.001$), Mbovis strain (0.06 % to 17.03 %, $p < 0.001$), the Ma strain (1.19 % to 28.24 %, $p < 0.001$), Mg strain (0.04 % to 14.61 %, $p < 0.001$) and Ms strain (0.05 % to 10.59 %, $p < 0.001$)

The highest viability rates were obtained with the lyophilized cultures of Ma (28.24 %), Mmm (17.85 %), Mb (17.03 %), Mg (14.61 %) and Ms (10.59 %); however, a viability of 5.91 % was observed with the Mmm strain conserved in glycerol and 4.75 % with the addition of the complete growth medium to the strain (Table 2).

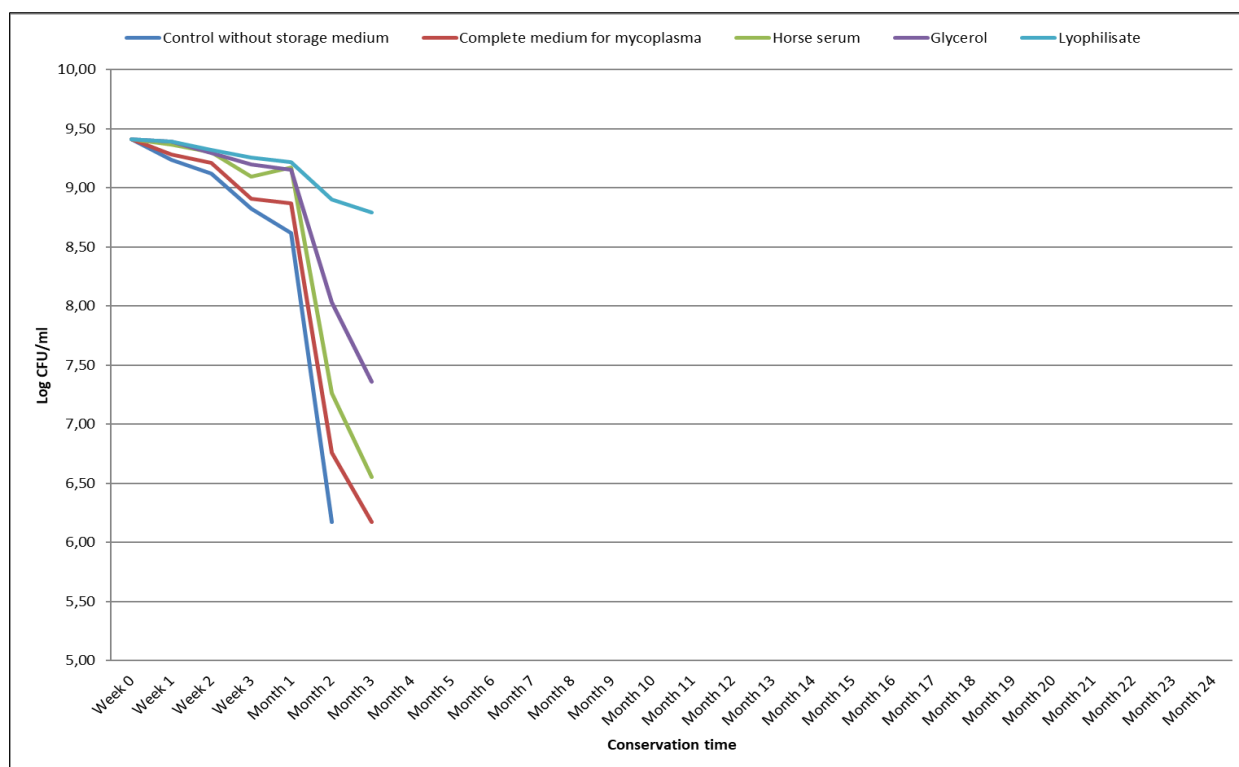


Figure 2 Mycoplasma viability at +4 °C. Mean loss (in log) of the titer of 5 strains of mycoplasmas (Mmm, Mb, Ma, Mg, Ms) during 24 months of storage in different preservatives

Table 2 Viability of mycoplasma strains at + 4 °C after 3 months

Mycoplasma strains		Mmm ^b	Mb ^c	Ma ^d	Mg ^e	Ms ^f
Initial titer (Log CFU/ml)		8.63	9.51	10.09	9.38	9.45
Control without Preservative (M1)	Log CFU/ml ^a	0.20	0.01	0.12	0.00	0.00
	Viability rate (%)	2.32	0.06	1.19	0.04	0.05
Complete medium (M2)	Log CFU/ml	0.41	0.01	0.33	0.00	0.01
	Viability rate (%)	4.75	0.09	3.27	0.04	0.07
Horse serum (M3)	Log CFU/ml	0.04	0.01	0.33	0.00	0.01
	Viability rate (%)	0.46	0.11	3.27	0.01	0.1
Glycerol (M4)	Log CFU/ml	0.51	0.01	0.37	0.08	0.03
	Viability rate (%)	5.91	0.11	3.67	0.85	0.32
Lyophilized cultures (M5)	Log CFU/ml	1.54	1.62	2.85	1.37	1.00
	Viability rate (%)	17.85	17.03	28.24	14.61	10.59

a colony format unity per mililiter ; bMycoplasma mycoides mycoides ; cMycoplasma bovis ; dMycoplasma agalactiae ; eMycoplasma gallisepticum ; fMycoplasma synoviae

3.3. Viability of mycoplasma strains at -20 °C

Overall, the mycoplasmas remained viable up to 24 months of storage at -20 °C with an approximate titer drop of 0.01 log per titration session. The lyophilized cultures lost approximately 1 log at M24 post storage while with preservatives such as horse serum and glycerol the loss was 1 to 2 log CFU / ml (Figure 3).

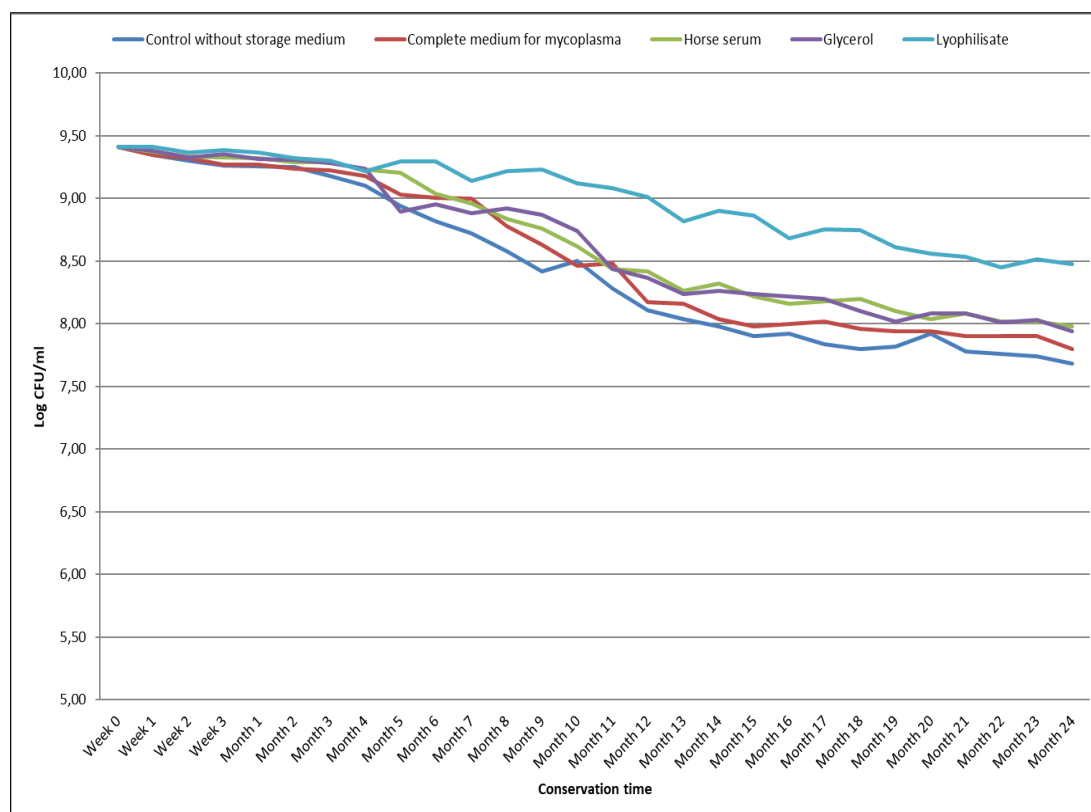


Figure 3 Mycoplasma viability at -20 °C. Mean loss (in log) of the titer of 5 strains of mycoplasmas (Mmm, Mb, Ma, Mg, Ms) during 24 months of storage in different preservatives

At -20 °C, after 24 months of storage, viability rates varied by storage medium with 81.60 % for the control without preservative, 82.88 % with the complete medium, 84.79 % with the addition of horse serum, at 84.36 % with glycerol as preservatives and 90.04 % with the lyophilized cultures. A non-significant effect ($p = 0.5229$) was observed between the viability rates of the mycoplasma strains and the storage media.

Viability rates varied by strain of mycoplasma and by preservative where lyophilized cultures, glycerol and horse serum maintained a rate above 90 % viability. The viability was 92.73 % to 99.34 % with Mmm in horse serum and lyophilized cultures, 84.12 % to 97.79 % for Mb with complete medium and lyophilized cultures, 79.26 % to 84.12 % for Ma without preservative and lyophilized cultures, 76.76 % to 83.15 % for Mg without preservative and lyophilized cultures and, 80.45 % to 86.80 % for Ms horse serum and lyophilized cultures (Table 3).

Table 3. Viability of mycoplasma strains at -20 °C after 24 months

Mycoplasma strains		Mmm ^b	Mb ^c	Ma ^d	Mg ^e	Ms ^f
Initial titer (Log UFC/ml)		8.63	9.51	10.09	9.38	9.45
Control without Preservative (M1)	Log CFU/ml ^a	7.20	8.10	8.00	7.20	7.90
	Viability rate (%)	83.46	85.17	79.26	76.76	83.62
Complete medium (M2)	Log CFU/ml	7.40	8.00	8.10	7.80	7.70
	Viability rate (%)	85.77	84.12	80.25	83.15	81.51
Horse serum (M3)	Log CFU/ml	8.00	8.20	8.40	7.70	7.60
	Viability rate (%)	92.73	86.22	83.22	82.09	80.45
Glycerol (M4)	Log CFU/ml	8.10	8.20	8.30	7.50	7.60
	Viability rate (%)	93.89	86.22	82.23	79.96	80.45

Lyophilized cultures (M5)	Log CFU/ml	8.57	9.30	8.49	7.80	8.20
	Viability rate (%)	99.34	97.79	84.12	83.15	86.80

a colony format unity per milliliter ; b *Mycoplasma mycoides mycoides* ; c *Mycoplasma bovis* ; d *Mycoplasma agalactiae* ; e *Mycoplasma gallisepticum* ; f *Mycoplasma synoviae*

3.4. Viability of mycoplasma strains at -85 °C

After 24 months of storage at -85 °C, the mean losses of titer of mycoplasmas in the whole were from 0.8 log (lyophilized cultures) to 1.06 log (horse serum, glycerol), the complete medium and controls with mean losses of 1.23 and 1.31 log CFU / ml. Cryopreservatives (glycerol and horse serum) and lyophilized cultures made it possible to maintain the mycoplasma strains viable with progressive losses over time (Figure 4).

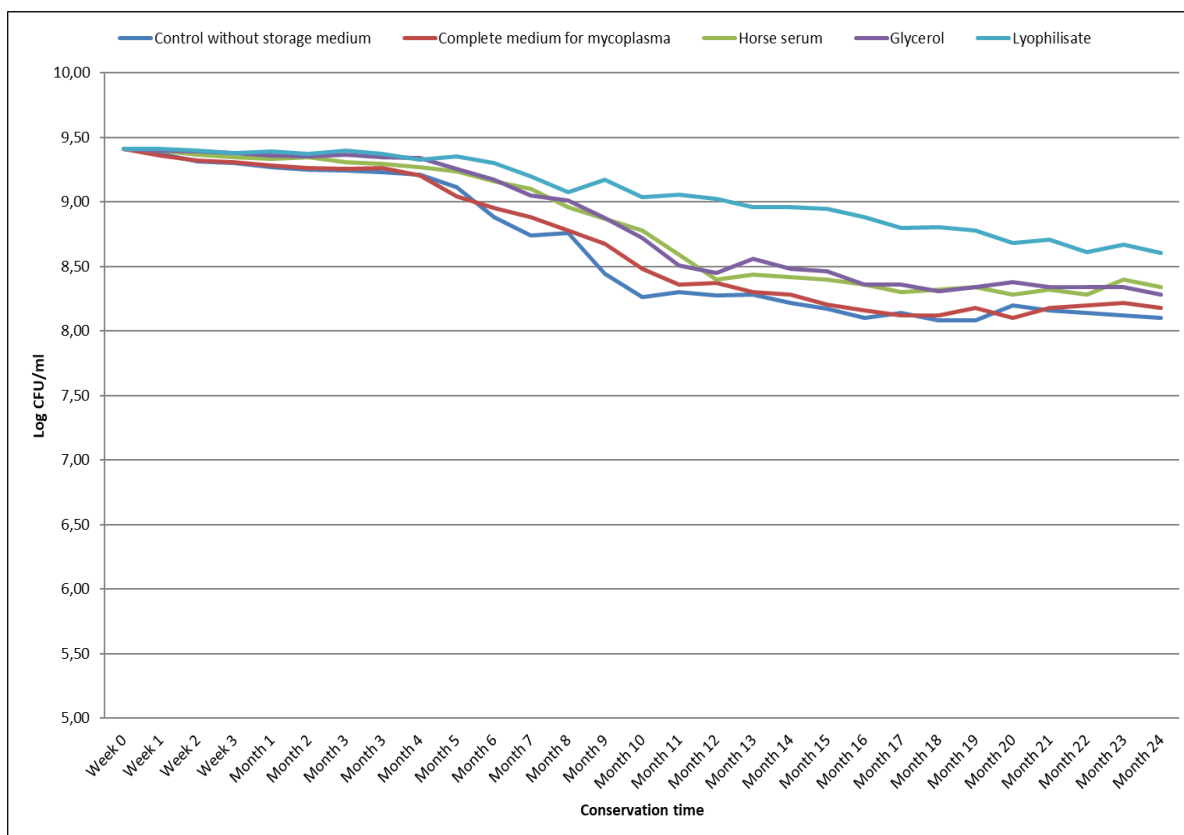


Figure 4 Mycoplasma viability at -85 °C. Mean loss (in log) of the titer of 5 strains of mycoplasmas (Mmm, Mb, Ma, Mg, Ms) during 24 months of storage in different preservatives

At -85 °C, non-significant overall viability rates ($p\text{-value} = 0.8035$) were obtained between the mycoplasma strains and the preservatives at 24 months of storage with respectively overall average viability rates of 86.06 %, 86.91 %, 88.61 %, 87.98 % and 91.44 % with control without preservative, complete medium, horse serum, glycerol and lyophilized cultures.

Viability rates of the mycoplasma strains after 24 months of storage under -85 °C depending on the preservatives were from 92.73 % (without preservatives) to 97.71 % (lyophilized cultures) for the Mmm strain, 86.22 to 92.53 % for Mb glycerol and lyophilized cultures, from 80.25 to 92.14 % for Ma without preservative and lyophilized cultures, from 85.29 to 88.48 % for Mg and from 85.74 % to 86.8 % for Ms (Table 4).

Table 4. Viability of mycoplasma strains at -85 °C after 24 months

Mycoplasma strains		Mmm ^b	Mb ^c	Ma ^d	Mg ^e	Ms ^f
Initial titer (Log UFC/ml)		8.63	9.51	10.09	9.38	9.45
Control without Preservative (M1)	Log CFU/ml ^a	8.00	8.30	8.10	8.00	8.10
	Viability rate (%)	92.73	87.27	80.25	85.29	85.74
Complete medium (M2)	Log CFU/ml	8.10	8.30	8.40	8.00	8.10
	Viability rate (%)	93.89	87.27	83.22	85.29	85.74
Horse serum (M3)	Log CFU/ml	8.20	8.40	8.70	8.20	8.20
	Viability rate (%)	95.05	88.32	86.19	87.42	86.8
Glycerol (M4)	Log CFU/ml	8.30	8.20	8.60	8.20	8.10
	Viability rate (%)	96.21	86.22	85.2	87.42	85.74
Lyophilized cultures (M5)	Log CFU/ml	8.43	8.80	9.30	8.30	8.20
	Viability rate (%)	97.71	92.53	92.14	88.48	86.8

a colony format unity per milliliter ; bMycoplasma mycoides mycoides ; cMycoplasma bovis ; dMycoplasma agalactiae ; eMycoplasma gallisepticum ; fMycoplasma synoviae

Significant differences in viability rates of Mmm, Mb, Ma, Mg and Ms were observed between different preservatives and temperature conditions. In general, all additives improved the viability rate of mycoplasmas compared to the control group. The highest viability rates of Mmm, Ma, Mg and Ms were observed at -85 °C with lyophilized cultures while for Mb this rate was observed with glycerol at -85 °C.

4. Discussion

The viability of vaccine and wild strains of mycoplasmas depends on the storage conditions, in particular temperature, preservatives and shelf life. With regards to temperature, to maintain cell cultures viability for extended periods, storage at -70 to -85 °C was much preferable especially with the addition of cryopreservatives such as dimethyl sulfoxide (DMSO) or glycerol. Our results showed that storage at -85 °C was preferable than at -20 °C which despite the loss of viability of the mycoplasmas during the freezing and thawing process seems better than storage at +4 °C intended for short-term storage (not more than 3 months). The lyophilized cultures of mycoplasmas allowed a better preservation of the viability whatever the temperature (+4, -20, -85 °C), which was also observed that the viability of the lyophilized cultures of mycoplasmas was stable for at least 34 months at +4 °C. In general, lyophilized cultures of various mycoplasmas were well preserved after long-term storage at 4 °C [17]. It is a fact that, at -85 °C, the metabolism process is completely stopped unlike -20 °C where the metabolism continues but at a very slow rate. Warming during thawing of strains affected negatively on the viability rate of frozen mycoplasmas. Rapid warming of frozen strains is preferable because it apparently prevents recrystallization of intracellular ice. Cryoprotective agents such as glycerol have been shown to be the most effective in protecting mycoplasmas cultures against freezing loss during the cooling process, possibly by reducing the fraction of frozen cellular water [18]. The effect of freeze-thaw conditions on the viability of Mb is a significant challenge for the preservation of these bacteria. The lack of peptidoglycan cell wall in mycoplasmas makes them susceptible to ice crystal formation during freeze / thaw processes [19]. It emerged from this study that glycerol led to an improvement in the survival of Mycoplasmas due to its bacteriostatic activity contributing to the inhibition of other bacterial proliferation [20]. The Mycoplasma strains tested in this study showed varying degrees of sensitivity to temperatures such as +37 °C, +4 °C, -20 °C and -85 °C. Cultures die quickly at +37 °C and at room temperature which would be incubation and not storage temperatures and at +4 °C where they would survive for 2 weeks in solid medium but less in liquid; on the other hand at -20 °C or less, the cultures will be able to survive for 6 to 12 months [21]. The viability of the microorganisms would be important with liquid nitrogen [22] but in the framework of routine diagnosis of mycoplasmosis from samples, the critical factors would be the storage temperature, the duration and the type of swab for the isolation of strains of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* [23]. Compared to *Mycoplasma bovis*, its viability in milk would depend on the storage temperature which for 5 days at +4 °C would reduce the number but the addition of glycerol (10 – 30 %) to the frozen milk samples improved the survival of *Mycoplasma bovis* whose detection will be maximized in fresh milk grown without storage [24]. The duration of viability of the mycoplasma strains under refrigeration temperature (+4 °C) did not exceed 3 months, unlike at temperatures of

-20 °C and -85 °C; this was confirmed by the study performed under anaerobic conditions where viable mycoplasmas were found with little or no reduction in titers after storage for 8 weeks at -30°C with a reduction in titers of 3 log₁₀ produced after 4 weeks of storage at +4 °C [25]. Freezing for 14 weeks did not significantly reduce *Mycoplasma bovis* titer compared to freezing for 2 weeks, and a second freeze-thaw cycle reduced *Mycoplasma bovis* count by approximately 0.5 log compared to a single freeze-thaw cycle [26]. Lyophilized cultures of *Mycoplasma* spp. stored at +4 °C had remained viable for 18 to 22 years and 82 % of *Mycoplasmas* initially lyophilized then reconstituted were viable after 16 years of storage at -70 °C [27]. Glycerol, in addition to its bacteriostatic power, is the main source of carbon and energy, especially for bacteria without cell walls of the genus *Mycoplasma* [28].

5. Conclusion

This study revealed that the lyophilized mycoplasmas without stabilizer, withstood storage at +4 °C for more than 3 months and that mycoplasma broths supplemented in storage medium (complete mycoplasma medium, horse sera, glycerol) could not remain viable beyond 3 months. It should also be noted that the addition of cryopreservatives such as glycerol to the cultures improved the viability of mycoplasmas. Temperatures of +37 °C and +4 °C were respectively for incubation (3 weeks) and short-term storage temperatures (3 months); on the other hand, for long-term storage the temperature of -85 °C is much more advantageous than -20 °C (>24 months in this study).

Compliance with ethical standards

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Disclosure of conflict of interest

This research work on mycoplasmas strains was conducted by Dr Amadou SERY without any conflict of interest along with his laboratory colleagues. Each member of the team had their full role in the successful conduct of this study. The main contributions of the authors are as follows : Conceptualization (SERY A., SIDIBE C.A.K., NIANG M.), Formal analysis (SERY A., SIDIBE C.A.K., NIANG M.), Funding acquisition (SERY A.), Investigation (SERY A., SIDIBE C.A.K., KONE M., SACKO B., NIANG M.), Methodology (SERY A., SIDIBE C.A.K., KONE M., SACKO B., NIANG M.), Supervision (SERY A., SIDIBE C.A.K., NIANG M.), Validation (SERY A., SIDIBE C.A.K., NIANG M.), Visualization (SERY A., SIDIBE C.A.K., NIANG M.), Writing – original draft (SERA A.), Writing – review & editing (SERY A., SIDIBE C.A.K., NIANG M., AWUNI J, AMANFU WILLIAM)

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