Original Article

Evaluation Of Different Techniques For Storage And Retrieval Of Microvials Stock Culture

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Abstract

Introduction: stocking of bacterial isolates is an integral part of clinical microbiology laboratory as it is used for research work, teaching and quality control purpose. preserving the strains while maintaining it phenotypic and genotypic characters is essential. Therefore, different stocking medias are used to keep them viable.

Aim: To check for the longevity and maintenance of phenotypic and genotypic characters by preserving the isolates in stocking medias like Peptone Glycerol Broth (PGB), Brucella Glycerol Both (BGB) and Skim Milk (SKM).

Materials and Method: this was a prospective analytical study where seven isolates along with three quality control strains were stocked in triplicates with cryobead based peptone broth with 15% glycerol, Brucella Broth (BB) with 15% glycerol and 10% SKM and stored at -80°C. The stock of each isolate in three different media is then revived at different intervals like monthly, quarterly and at 10th month to check for their viability and phenotypic characters and these are then compared with the readings taken before preservation.

Results: revival rate of PGB, BGB and SKM was 100% and 99% and 90 % respectively when revived monthly. Quarterly revival rate was 100%, 96.60% and 93.30% for PGB, BGB and SKM respectively. Phenotypic characters and antibiotic susceptibility pattern were maintained by all three media after repeated as well as single freeze thaw.

Conclusion: peptone broth with 15% glycerol containing cryobeads can be used as an effective preparation for stock culture maintenance of non-fastidious bacteria and yeast.

INTRODUCTION:

It is important to stock bacterial isolates for extended periods as it is required for research, teaching and quality control purpose. Specific storage techniques are required to maintain the stock cultures for extended periods [1]. Organisms tend to remain viable for few days to years and the ability of the organism to remain viable during storage depends upon factors like temperature, pH, oxidative stress and their own special metabolic need [3].

Effective storage is defined by the ability of an organism to maintain a viable state free of contamination and without changes in its genotypic or phenotypic characteristics. Bacterial stocking can be done by different methods like cold storage, drying and freeze drying (lyophilisation) etc. [2, 3].

Storage practices should be easy and cost effective and the most commonly used method in clinical laboratories for preservation is cold storage under ultra-low freeze temperature (-20ºC and -80ºC). To protect microorganisms from damage during the freezing process, during storage, cryoprotective agents are often added to the culture suspension to avoid any damage to stock culture but repeated freeze thaw cycles are to be avoided as repeated freeze- thaw cycles change the phenotypic and genetic makeup of the microorganisms [4].

To maintain stock cultures different basal media can be used e.g., peptone glycerol broth, brucella broth, skimmed milk, Brain heart infusion etc along with cryoprotective agents like DMSO, glycerol etc.
Cryobeads can be added to the stock fluid for adsorption of bacteria and fungi. It gives an advantage that only one bead is taken out at the time of subculture mixed with peptone water and cultured and the whole stock is not thawed thus avoiding any contamination and other changes [2, 5].

Our study compared three basal medias to check for their efficacy: Peptone Glycerol broth with 15% glycerol, Brucella broth with 15% glycerol and 10% skim milk. The isolates were checked for their longevity and phenotypic characters before and after preservation.

MATERIALS AND METHODS:

This prospective analytical study was conducted in the bacteriology laboratory of Department of Microbiology of Acharya Shri Chander College of Medical Sciences and Hospital, Jammu. The study began in October 2021 and was conducted for a period of 10 months. Before conducting the study, clearance was taken from Institutional Ethical Committee (IEC). Ten different isolates were included in the study and their phenotypic characters as well as their Antibiotic susceptibility pattern was noted before stocking the cultures. For each isolate three cryovial preparations were made for each stocking media i.e., Peptone Glycerol Broth (PGB), Brucella Broth (BB) and Skimmed Milk (SKM). Reproducibility of the morphological, biochemical and AST results before and after storage was assessed by same methods during each revival. Media was prepared and sterilised according to the manufacturer's instructions.

Cryobeads were added to each stock preparation.

The isolates included were as follows:

1. *Escherichia coli* ATCC 25922
2. *Staphylococcus aureus* ATCC 25923
3. *Enterococcus faecalis* ATCC 29212
4. *Candida albicans*
5. *Escherichia coli*- AmpC producer
6. *Klebsiella pneumoniae*- confirmed ESBL producer
7. *Methicillin resistant Staphylococcus aureus* (MRSA)
8. Multidrug resistant *Pseudomonas aeruginosa*
9. Vancomycin resistant *Enterococcus faecalis* (VRE)
10. Multidrug resistance *Klebsiella pneumoniae.*

The phenotypic characters as well as AST pattern was noted before stocking and then at stipulated time interval during the study period.

Bacterial identification: Cultures were done using Blood agar and MacConkey agar and their phenotypic characters were noted.

Smear preparation and Staining: A smear was then prepared using the growth on the plate that we obtained after overnight incubation and it was stained using Gram Staining technique and observed under oil immersion lens and an account was made of all the readings observed.

Biochemical tests used to detect the organisms:

- Indole production test
- Methyl Red test
- Urease production test
- Mannitol motility
- Phenyl pyruvic acid test
- Triple sugar iron test
- Oxidative/Fermentation test
- Sugar fermentation test
- Citrate utilization test
- Nitrate reduction test
- Catalase test
- Oxidase test

Antibiotic Sensitivity Testing:

The antibiotic susceptibility of the bacterial isolates was tested by Kirby- Bauer disc diffusion method on Muller-Hinton agar by following guidelines of Clinical and Laboratory Standards Institute (2021).

Antibiotics for **Gram- negative** isolates that were tested in our study included Amoxicillin/clavulanic acid, Ampicillin, Piperacillin-tazobactam, Amikacin, Cefuroxime, Ceftazidime, Cefoxitin, Cefepime, Ciprofloxacin, Cotrimoxazole, Gentamicin, Imipenem, Nitrofurantoin, Tobramycin, Tetracycline, Norfloxacin, Fosfomycin, Aztreonam

Antibiotics for **Gram- positive** isolates that were tested in our study included:
Erythromycin, Clindamycin, Oxacillin, Penicillin, Cotrimoxazole, Linezolid, Tetracycline, Vancomycin, Chloramphenicol, Ciprofloxacin, Gentamycin, Cefoxitin.
Antifungals for *Candida albicans:* Fluconazole, Itraconazole, Amphotericin B.

Detection of ESBL was done by Disc potentiation test:

Screening: Strains resistant to cefpodoxime (10µg), Ceftazidime (30µg) and Cefotaxime (30µg).

Confirmatory: Disc potentiation test was carried out by using ceftazidime 30µg and ceftazidime 30µg + clavulanic acid 10µg disks

Interpretation: Isolates showing ≥ 5mm inhibition zone enhancement around ceftazidime 30µg + clavulanic acid 10µg disks vs ceftazidime 30µg alone were labelled as ESBL producer [4]

Detection of AmpC

Screening: Strains resistant to Cefoxitin
Confirmatory test: Was done by using cefoxitin disc(30µg) and Cefoxitin disc (30µg) + Phenyl Borinic Acid (400µg) on Muller Hinton agar plate already inoculated with the test strain and was incubated overnight at 37°C.

Interpretation: Organism that demonstrates a defined increase (≥5mm) in zone diameter around the antibiotic disk with added boronic acid is considered to be AmpC producer [4]

Stock Media Preparation:

1) **Peptone Glycerol Broth (PGB):** 3ml of cryovial was taken to which 12 cryobeads were added. 150 µL of glycerol was added to 850 µL peptone water 1% (HiMedia) and autoclaved at 121°C for 15 minutes. Heavy growth of overnight culture was added to it with the help of a cotton swab.
2) **Brucella Broth (15% glycerol added):** 3 ml of cryovial was taken to which 12 cryobeads were added. 150 µL of glycerol added to 850 µL BB (HiMedia) and autoclaved at 121°C for 15 minutes. Heavy growth of overnight culture was added to it with the help of a cotton swab.
3) **10% Skim milk-stock preparation:** 3 mL cryovials containing 12 cryobeads were sterilised by autoclaving at 121°C for 15 minutes. 10% skim milk (HiMedia) was prepared and autoclaved at 121°C for 5 minutes. Heavy suspension of the individual isolates was added in 1 mL of skim milk with the help of sterile cotton swab. The suspension was transferred to cryovials with beads.

Revival of stock: Revival was done is three different patterns:
Three sets were made of each glycerol containing basal media against each isolate:
Revival of the stock was done monthly, after every three months and one set was left undisturbed and was revived at the end of 10 months i.e., set 1 was checked for isolates every month for 10 months, set 2 was revived after every 3 months and set 3 was revived at the end of 10 months.
Cryovials were taken out of the freezer (-80ºC) and one cryobead was removed from it and again cryovial was immediately put back in freezer to avoid any contamination. The cryobead was put in 1ml of peptone water and after incubation at 37ºC for 2-3 hours it was subcultured on blood and MacConkey agar.
Longevity was checked by presence or absence of growth and difference in growth before and after stock culture. Phenotypic characters were assessed by biochemical reactions and antibiogram pattern for each revived strains as per standard guidelines.

RESULTS:

In our study 10 isolates were stocked over a period of 10 months and they were made in triplicates accounting for 30 stocks per method. Isolates revived were checked for their growth and time taken to revive.

		A	B	C	D	E	F	G	H	I	J	Revival% age
Revival pattern	Preparation											
Monthly (n=10)	PGB	10	10	10	10	10	10	10	10	10	10	100%
	BGB	10	10	10	10	10	10	10	9	10	10	99%
	SKM	10	7	8	8	10	10	9	10	9	9	90%
Quarterly (n=3)	PGB	3	3	3	3	3	3	3	3	3	3	100%
	BGB	3	3	3	3	3	3	3	3	2	3	96.60%
	SKM	3	3	3	3	3	3	2	2	3	3	93.30%
10th Month (n=1)	PGB	1	1	1	1	1	1	1	1	1	1	100%
	BGB	1	1		1	1	1	1	1	1	1	100%
	SKM	1	1	1	1	1	1	1	1	1	1	90%

Table 1: Revival percentage of individual isolates stored in all three preparations under monthly, quarterly and after 10 months patterns. A- *Escherichia coli* ATCC 25922, B-*Staphylococcus aureus* ATCC 25923, C- *Enterococcus faecalis* ATCC 29212, D- *Candida albicans,* E- *Escherichia coli* (Amp C), F- *Klebsiella pneumoniae* (ESBL), G-MRSA, H-Pseudomonas aeruginosa (MDR), I- Vancomycin resistant *Entrococcus faecalis, J- Klebsiella pneumonia* MDR. n-Number of times revival performed under this pattern for individual isolates.

As seen in Table 1 the percentage of revival was calculated for each isolate in all three stocking preparations at different intervals. i.e., each isolate was checked for its longevity and phenotypic characters in different media at different time intervals (monthly, quarterly and at 10th month).

In all 10 isolates no contamination could be seen in any stocked cryobead preparation.
There was 100% and 99% revival for PGB and BGB respectively, with confluent growth irrespective of the frequency of revival.
In SKM less growth was seen for six isolates with monthly revival after 8th and 9th month (*Staphylococcus aureus, Enterococcus faecalis, Candida albicans,* Methicillin Resistant *Staphylococcus aureus*, Vancomycin resistant *Enterococcus faecalis*, Multidrug resistance *Klebsiella pneumoniae).* No growth was seen for two isolates (Methicillin Resistant *Staphylococcus aureus* and Multidrug Resistant *pseudomonas aeruginosa*) at quarterly interval and for one isolate (Methicillin Resistant *Staphylococcus aureus*) at 10th month. Therefore, it was clear that the efficacy of SKM was slightly low in terms growth revival.
There was no change in the biochemical reactions of all the isolates revived and also there was no significant drift in mapping of Antibiotic resistance pattern in all three stocking media. Results were similar to the pre storage condition and all these isolates were tested in 14 (10+3+1) episodes of revival.
Freeze thaw had no effect on resistance pattern of the isolates.

DISCUSSION:

Preservation of stock cultures has become an important part of microbiology laboratories as it is used for research, teaching and epidemiological purpose [2].
Out of many methods available for long term preservation of isolates, cryobead method of preservation is considered the best since the chances of contamination are least and complete freeze thaw is not required [6]. Results from the past studies too showed that cryobead method of preservation gives excellent revival rate. Similar results were observed in our study. In our study it was also observed that, using cryobeads makes it easy and mess free as only one bead is used at one time and full vial is not freeze thawed.
Cryoprotective agents like glycerol and SKM are well known for their effective revival rate. In our study, Glycerol based combination were found to have better cryoprotective effect as compared to SKM. In this study, the revival rate of isolates for Glycerol based agents was 100% at monthly, quarterly and at 10th month whereas for SKM it was 90% for monthly revival, 93.3% quarterly and 90% at 10th month. Similar results were found in other studies testing fungal and bacterial cultures [2,6,7]. In this study, it was observed that the efficacy of SKM mainly decreased in the later months and was mainly seen for MRSA.
The phenotypic characters and Antibiotic sensitivity pattern of all the isolates were similar to the readings that were taken before storage. The ATCC strains tested against common drugs retained their QC ranges. Variation in zone diameter of each drug tested against specific organisms was ±3mm with no discrepancy observed.
All three preparations used in this study showed good revival rates but the best amongst them were PGB and BGB as compared to SKM. The cost of preparation of BGB is high as compared to PGB and SKM is the cheapest.

CONCLUSION:

Current study showed that PGB preparation could be used as an alternative to BGB and SKM in cryobead stocking method for bacterial and yeast isolates as it supported long term viability with 15% glycerol as compared to SKM. This study also showed that glycerol-based preparations had a better cryoprotective effect than SKM preparation. Peptone based stock preparation are easily available, cheap, less time-consuming procedure in diagnostic laboratories and hence can be the preferred agent for long term cryobead preservation at -80ºC.

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