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EVALUATION OF DIFFERENT CRYOPROTECTIVE AGENTS IN MAINTENANCE OF VIABILITY OF *HELICOBACTER PYLORI* IN STOCK CULTURE MEDIA

Daryoush Davoudi Oskouei [1], Neslihan Bekmen [1], Hülya Ellidokuz [2], Özlem Yılmaz [1*]

[1]Department of Microbiology and Clinical Microbiology, Dokuz Eylül University, İnciraltı – 35340, İzmir, Turkey, [2]Faculty of Medicine, Public Health Division of University Hospital, Dokuz Eylül University, İnciraltı – 35340, İzmir, Turkey

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ABSTRACT

Four different cryoprotective supplemented stock media were evaluated for maintaining better survival and recovery of *H. pylori* type strain NCTC 11637 at two different maintenance temperatures of -20°C and -80°C after one month preservation as frozen stocks.

The spread plate colony count method was used to investigate the recovery rate of *H. pylori* from equally inoculated bacterial suspensions in differently prepared stock cultures.

After the preservation of *H. pylori* for one month in different cryoprotectant-supplemented stock media, the recovery rates for -20°C obtained for stock cultures supplemented with dimethyl sulfoxide (DMSO), polyethylene glycol (PEG), glycerol and glycerol+sucrose, as well as controls with and without human serum alone were 7.13, 6.97, 7.93, 7.99, 6.95 and 0.0 log CFU/ml, respectively. Maintenance of bacteria at -80°C gave statistically higher recovery rates compared to preservation at -20°C with the values of 8.55, 8.24, 8.59, 8.66, 8.01 and 0.0 log CFU/ml for these above mentioned stock cultures. The stock cultures supplemented with glycerol+sucrose and glycerol showed the highest recovery rates, 7.99 and 7.93 for -20°C vs. 8.66 and 8.59 for -80°C respectively, which were statistically different from the others.

Our study revealed that *H. pylori* type strain NCTC 11637 could be better preserved at -80°C than -20°C. The best stock media which supported viability or culturability of bacteria were brain heart infusion broth (BHI)+glycerol+human serum and BHI+glycerol+sucrose+human serum, where the latter yielded the higher recovery rate.

Key words: *Helicobacter pylori*; recovery rate; stock culture media; cryoprotective agents

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram-negative spiral-shaped bacterium, which is closely associated with gastroduodenal diseases such as gastritis, peptic ulcers and gastric cancer. The isolation of the organism from biopsy specimens and maintenance of bacterial isolates without loss of culturability are both very important in conducting antimicrobial susceptibility testing as well as studying virulence factors (5, 15).

***Corresponding Author. Mailing address:** Dokuz Eylül University, School of Medicine, Department of Microbiology and Clinical Microbiology, İnciraltı, 35340, İzmir, Turkey.; Tel: +90 (232) 412 4506 Fax: + 90 (232) 259 0541.; E-mail: ozlem.yilmaz@deu.edu.tr

H. pylori is a fastidious organism; it has a low growth rate and its culture requires nutrient-rich media (13). *H. pylori* is also very sensitive to environmental stresses, such as oxygen, which may cause the bacterium to change from the bacillary form to a non-culturable or so-called coccoid form, hence making the culture of the organism very hard or impossible. Coccoid formation is an active process switched on by the bacterium as a protective mechanism (4) and also can mediate genetic exchanges between neighboring organisms (19).

Unlike other bacteria, the storage of *H. pylori* is faced with many problems. Several methods have been developed to preserve *H. pylori.* Early studies have shown that freeze-drying is not a suitable method because it harms bacteria. Lyophilizing without freezing using 20% L-glutamic acid has been recommended, but this method is too cumbersome for routine use (2).

Although freeze-drying of a dense *H. pylori* suspension prepared in skim milk has yielded successful recovery of bacteria, this method is not reliable for good recovery after subculture on solid media. Furthermore, not every laboratory can afford equipment to freeze-dry *H. pylori* directly from the liquid state (23). Freezing methods such as storage in 10% mucin, fetal bovine serum, or cryoprotective liquids (e.g., 25% glycerol) at -193°C or -70°C have been described to guarantee a viability of 77 - 90%. Preservation at -193°C has not been more successful than at -70°C (2). Some researchers have also shown that *H. pylori* can be safely stored in its natural environment, the gastric mucosa, for a long time. Glycerol-containing media such as cysteine-Albimi, Brucella broth or skim milk have been proposed for optimal preservation of *H. pylori* in gastric mucosa as well as bacterial isolates at -70°C (9).

Like in other living cells, ice crystals formed in *H. pylori* during freezing cause damage to the organism. Therefore, the easiest and the most commonly accepted method to maintain bacterial isolates for a long time generally involves deep freezing the bacteria using a cryoprotectant. It decreases the need for repeated subculturing which can lead to contamination, genotypic or phenotypic alterations, as well as spontaneous autolysis (3, 8). However, some of these cryoprotective agents such as glycerol and DMSO may be toxic for a living organism and also may fail to afford enough protection during freezing (7). Several studies have also addressed the influence of temperature as well as morphology and amount of initial inoculum on viability and recovery of *H. pylori* from stock cultures (2, 21). In another recent study, sucrose was shown to be a promising in preserving human sperm (11), so sucrose may have such a protective effect on the viability of *H. pylori* strains.

In our study, the effectiveness of these different cryoprotective agents in the maintenance of *H. pylori* during freezing was evaluated to determine the best protective agent for the viability or culturability of a standard reference strain of *H. pylori,* NCTC 11637 (*cagA, vacA*, *oipA* and *babA* positive), from frozen stock cultures. We used the spread plate colony count method to detect the recovery rate of *H. pylori* from stock cultures after one-month maintenance at two different temperatures of -20°C and -80°C.

MATERIAL AND METHODS

H. pylori inoculum preparation

H. pylori NCTC 11637, previously preserved at -80°C in stock medium (BHI broth supplemented with 20% glycerol), was used in this study. It was subcultured on *H. pylori* selective agar medium consisting of Columbia blood agar medium (Oxoid, United Kingdom) supplemented with *Helicobacter pylori* Selective Supplement (DENT, Oxoid, United Kingdom), 10% filter-sterilized, heat-inactivated fresh human serum and 0.3% activated charcoal (6). The plates were incubated at 37°C for 72 h under microaerobic conditions using the GasPak EZ Campy Container System (Becton Dickinson, USA). Bacterial cultures were confirmed as *H. pylori* by positive urease, oxidase and catalase tests as well as Gram-negative spiral morphology. The bacterial suspension equivalent to a McFarland 3 standard (~ 10^9 CFU/ml) was prepared from a 72 h culture of *H. pylori* standard strain in phosphate-buffered saline (PBS) (pH 7.4) and used as an inoculum for stocking.

Before inoculation, a smear was prepared and stained with a modified Gram's staining method to ensure that almost all bacteria had typical spiral morphology and that there were no bacterial contaminations. Bacterial suspension was also checked by a colony count method to ensure that the inoculum consisted of ~ 10^9 CFU/ml.

Preparation of stock media

20% glycerol supplemented medium: For preparation of 20 ml stock medium, 0.74 g brain heart infusion broth (BHI) (Oxoid, United Kingdom) and 4 ml glycerol (Baker Analyzed, USA) were separately dissolved in 8 ml and 6 ml distilled water, respectively, and autoclaved at 121°C for 15 min. The two were then mixed together, and 2 ml of filter-sterilized (through a disposable filter of 0.45 µm pore diameter) and heat-inactivated (56°C for 30 min) fresh human serum (Blood Bank, Dokuz Eylül University Hospital, Izmir) were added. The stock medium was dispensed into sterile Eppendorf tubes, each 0.5 ml, and stored at 4°C until used.

5% polyethylene glycol (PEG) supplemented medium: BHI broth, 0.74 g, was dissolved in 17 ml distilled water. After autoclaving at 121°C for 15 min, 1 ml of filter sterilized PEG (Merck, USA) (through a disposable filter of 0.45 µm pore diameter), and 2 ml filter-sterilized heat inactivated fresh human serum were added. Stock medium was dispensed into sterile Eppendorf tubes and stored as mentioned above.

5% dimethyl sulphoxide (DMSO) supplemented medium: This was prepared as mentioned for PEG, except 1 ml DMSO (Sigma-Aldrich, USA) was used instead of PEG.

0.3 M sucrose supplemented medium: BHI broth, 0.74 g, and 4 ml glycerol in 6 ml distilled water were separately autoclaved at 121°C for 15 min. A solution of 2 g sucrose (Merck, USA) in 2 ml distilled water (0.3 M) was filter-sterilized (through a disposable filter of 0.45 µm pore diameter). A volume of 2 ml filter- sterilized, heat-inactivated fresh human serum was added to the mixture. Stock medium was dispensed into sterile Eppendorf tubes and stored as mentioned above.

Bacterial stocking procedures

Stock media previously maintained at 4°C were brought to room temperature and 100 µl of bacterial suspension was introduced into 0.5 ml of each of the storage media. After vortexing, all the stock cultures were frozen at -80°C and -20°C within 15 min.

BHI broth with and without human serum containing *H. pylori* bacterial suspensions were included as control A and B groups in this study. A total of 120 stock culture samples were studied under two different maintenance temperatures of -80°C and -20°C for one month.

Plating for determination of colony forming units (CFU)

After thawing the *H. pylori* stock culture samples, the vials were vortexed, and 10 µl of each cryopreserved stock sample were serially diluted 10^4-fold into 990 µl of sterile PBS (pH 7.4). Next, 10 µl of each diluted suspension were streaked onto *H. pylori* selective agar medium for the determination of the number of CFU by the spread plate colony count method. The plates were incubated at 37°C for 5 days under microaerobic conditions (GasPak EZ Campy). Colony counts per plate (CFU/ml) were calculated as: number of colonies x [1/dilution] per ml.

Dilution factor was estimated by preparing 10^2-, 10^3- and 10^4 -fold dilutions for each type of stock culture. Finally, a 10^4-fold dilution which yielded countable bacterial colonies on agar plates was selected and applied for all stock cultures. Recovery rate (log CFU/ml) was also calculated and analyzed for each plate.

Statistical analysis

The Mann-Whitney U test (Asymp. Sig., 2-tailed) was used for the analysis of differences between two independent nonparametric samples: the recovery rates of *H. pylori* from different stock cultures as well as different maintenance temperatures. The differences were considered statistically significant if $p<0.05$.

RESULTS

The averages obtained for 10 µl of each type of stock culture with 10^4-fold dilutions containing 100 µl of *H. pylori* NCTC 11637 suspension equivalent to a Mc Farland 3 standard were used. Recovery rates as well as the log reduction determined relative to the initial inoculum were compared between the different cryopreserved stock cultures. The viability or recovery rate of *H. pylori* for the various cryoprotectant-supplemented stock cultures after 1 month maintenance at two different temperatures were given as the mean log CFU/ml ± SD (Table 1). All types of stock cultures but not stock control samples A (without human serum) yielded culturable *H. pylori* colonies after one month maintenance at -20°C and -80°C. The differences were statistically significant (p=0.000) while stock control samples B (with human serum) had almost similar colony count numbers.

Table 1. Recovery rate of *H. pylori* from stock cultures using different cryoprotective supplemented stock media

Stock medium	No. of samples	Maintenance temperature	Mean log CFU/ml ±SD
BHI[(a)]+5% DMSO[(b)]+10%HS[(c)]	5*	-20	7.15±0.24
	10	-80	8.55±0.18
BHI+5%PEG[(d)]+10%HS	8*	-20	6.97±0.20
	9*	-80	8.24±0.20
BHI+20%glycerol+10%HS	10	-20	7.93±0.43
	10	-80	8.59±0.32
BHI+20%glycerol+0.3 M sucrose+10%HS	10	-20	7.99±0.36
	10	-80	8.66±0.21
BHI (control A)	10	-20	0.0±0.0
	10	-80	0.0±0.0
BHI+HS (control B)	5*	-20	6.95±0.14
	9*	-80	8.01±0.17
	106		

(a); Brain heart infusion broth
(b); Dimethyl sulfoxide
(c); Human serum
(d); Polyethylene glycol
*; Due to contamination as well as some failures in *H. pylori* recovery at a defined dilution, a number of samples were excluded from 20 prepared for each type of stock culture.

The recovery rate of *H. pylori* as mean log CFU/ml for all stock cultures, except control A samples, was higher with freezing at -80°C than at -20°C [BHI+DMSO+human serum (HS), 8.55± 0.18 vs. 7.15±0.24; BHI+PEG+HS, 8.24±0.20 vs. 6.97±0.20; BHI+glycerol+HS, 8.59±0.32 vs. 7.93 ±0.43; BHI+glycerol+sucrose+HS, 8.66±0.21 vs. 7.99±0.36; BHI+HS (control B), 8.01±0.17 vs. 6.95±0.14]. The differences were statistically significant for these stock cultures (p=0.001, p=0.000, p=0.004, p=0.000, p=0.001, respectively) (Figure 1).

While there was a nearly 1 log reduction in bacterial colony counts for glycerol and glycerol+sucrose supplemented stock cultures maintained at -20°C, the three other stock cultures (DMSO, PEG and control B) showed almost 2 log reduction in colony count number. The control B stock culture samples were also able to maintain bacterial culturability as high as in DMSO and PEG supplemented stock cultures. The

glycerol+sucrose and glycerol supplemented stock media could support the viability or culturability of *H. pylori* at -20°C for one month, better than other cryoprotective agents (Figure 2). The differences between recovery rate of bacterial colonies of these stock cultures and others were significant for the following media: glycerol and DMSO (p=0.005); glycerol and PEG (p=0.000); glycerol and control A (p=0.004); glycerol+sucrose and DMSO (p=0.003); and glycerol+sucrose and PEG (p=0.000). However, the higher recovery rate observed for glycerol+sucrose at -20°C was not found to be statistically significant from that of glycerol-supplemented stock cultures (p=0.970).

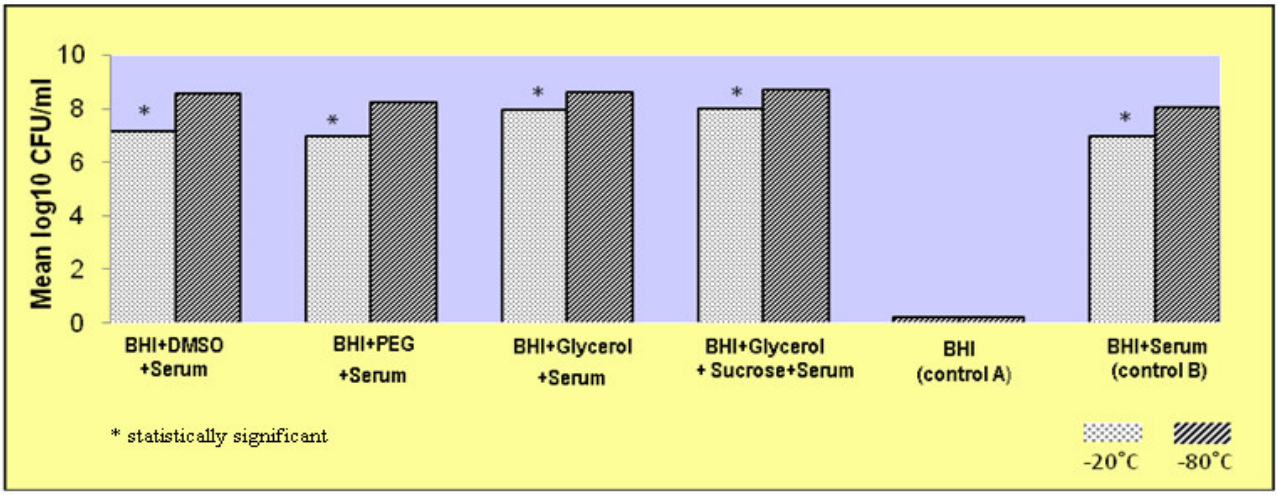

Figure 1. The recovery rate of *H. pylori* NCTC 11637 standard strain after one month of maintenance as various stock cultures at -20˚C and -80˚C, determined by spread plate colony count method.

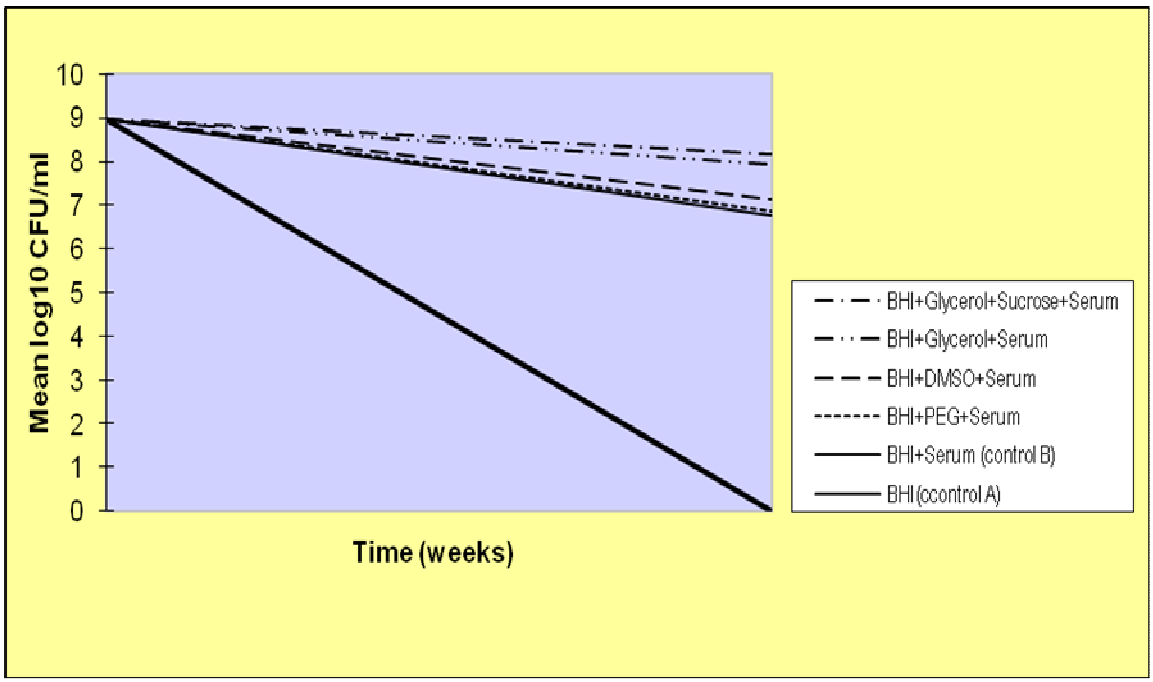

Figure 2. The log10 changes in viability of *H. pylori* NCTC 11637 standard strain maintained as various stock cultures for one month at -20˚C, determined by the spread plate colony count method.

Preservation at -80°C resulted in less log reduction in bacterial colony counts compared to preservation at -20°C. The log reduction in bacterial colony counts for stock cultures supplemented with DMSO, PEG, glycerol and glycerol+sucrose were 0.4, 0.71, 0.36 and 0.29, respectively. While there were no colonies formed with control A stock culture samples, which showed 8.95 log reduction, the control B stock culture samples could maintain the culturability of bacteria as high as 8.01 and showed about 1 log reduction after one month preservation of bacteria at -80°C. Stock cultures supplemented with glycerol+sucrose, glycerol and DMSO (in descending order) could support the viability or culturability of *H. pylori* at -80°C, better than other cryoprotective agents, for one month (Figure 3). The differences between recovery rate of bacterial colonies from these stock cultures and others were statistically significant for the following media: glycerol+sucrose and control A (p=0.000); glycerol and control A (p=0.000); glycerol and PEG (p=0.035); DMSO and PEG (p=0.008); DMSO and control A (p=0.000). Despite the higher recovery rate observed for glycerol+sucrose-supplemented stock cultures, the differences between three stock cultures were not found to be statistically significant for the following media: glycerol and glycerol+sucrose (p=1.000); DMSO and glycerol+sucrose (p=0.218); and DMSO and glycerol (p=0.579).

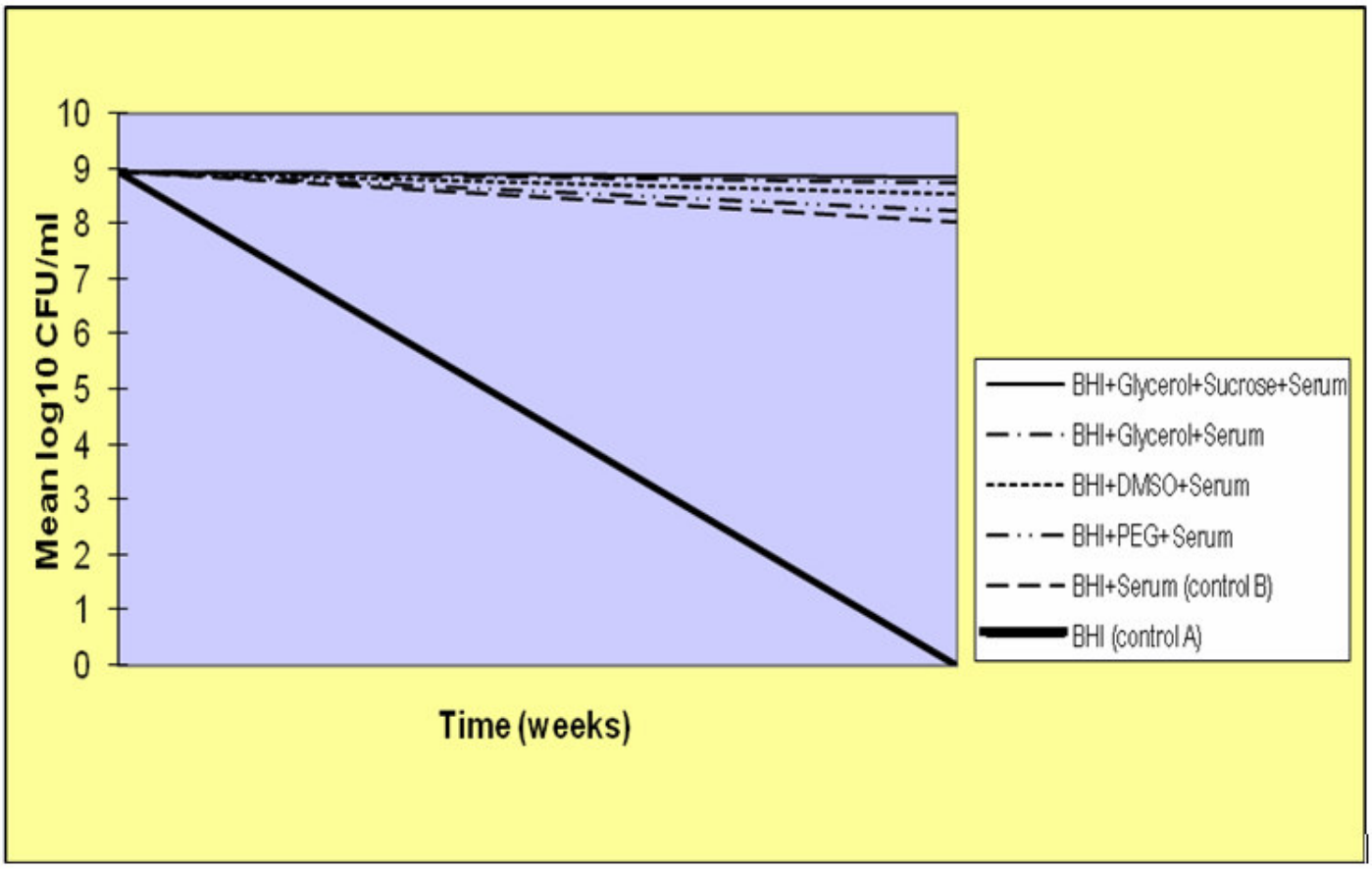

Figure 3. The log10 changes in viability of *H. pylori* NCTC 11637 standard strain maintained in various stock cultures for one month at -80˚C, determined by the spread plate colony count method.

DISCUSSION

Culture and maintenance of bacterial strains without loss of culturability is crucial for carrying out antimicrobial susceptibility testing as well as for studying virulence factors. Studies conducted on the preservation of *H. pylori* may also be useful in developing transport media for optimal recovery of the organism from gastric biopsy specimens (10, 15). Despite the availability of many methods, cryopreservation of living bacteria at a low temperature is the most commonly accepted way to stabilize cultures for long-term storage. It decreases contamination and genotypic and phenotypic variations, and eliminates the need for media that are expensive and require time- consuming preparation (3, 7, 21). The purpose of this study was to evaluate different cryoprotectants in preserving *H. pylori* during freezing and to determine the more protective agent in survival or recovery of *H. pylori* from frozen stock cultures. Our results obtained from different cryoprotectant-supplemented stock cultures showed that *H. pylori* type strain NCTC 11637 could be better preserved at -80°C than -20°C. At least 0.3 - 1 log reduction in bacterial recovery were seen for all stock cultures maintained at -20°C and -80°C for one month, respectively. It has been shown that storage temperatures below –130°C were critical for the long-term stability of bacterial cultures, because this is the point at which ice crystal formation stops. At temperatures above –130°C, ice crystals continue to grow and reform (3). Ohkusa et al. demonstrated that unlike with other intestinal bacteria, the preservation of *H. pylori* strains in saline at 4°C, -20°C and even at -80°C results in failure of bacterial recovery and infectivity in Mongolian gerbils due to changes from the bacillary to coccoid form (16).

Some species of bacteria including *H. pylori* enter a physiologically dormant phase or so-called viable but non-culturable (VBNC) state when faced with environmental stresses. Stresses such as elevated osmotic levels and oxygen concentration, low nutrients and incubation at tempratures unsuitable for growth could make the organism enter this state. It has been shown that these non- culturable coccoid forms differ from culturable forms which undergo starvation and have significantly low metabolism (17). These results showed that agents were needed to be added into stock media to protect bacteria from freezing stress.

In spite of reports about the toxicity of glycerol (7), our study showed that this agent had a more protective effect against freezing stress. Glycerol-supplemented stock media were significantly more protective at -20°C and -80°C in comparison to other cryoprotectant-supplemented stock cultures. This effect was also more significant when stock cultures were preserved at -80°C rather than at -20°C. Our results with glycerol were similar to those of Han et al. in which *H. pylori* was preserved with greater viability at -70°C than -20°C when glycerol-containing media such as skim milk, Brucella broth and cysteine-Albimi broth were used. Lactose which is present in skim milk might have contributed to improving the viability of *H. pylori* (9) by a similar mechanism, with regard to sucrose in our study.

The mechanism by which intracellular glycerol confers resistance to freezing stress has been explained by Izawa et al. They found increased levels of intracellular glycerol in knockout mutants of glycerol dehydrogenase genes in *Saccharomyces cerevisiae*, resulting from a decrease in glycerol dehydrogenase activity in comparison to wild types. This increase in intracellular glycerol confers more tolerance to freeze-thawing stress. Despite preventing ice crystal formation, the effects of increased intracellular glycerol on freeze tolerance may arise partly from changes in lipid composition (12, 21).

The growth supportive feature of bovine and human serum has been described by Shibayama et al. and Paik et al., respectively. By heating at 56°C for 30 min and inactivating complement which is the major bactericidal substance in bovine as well as human serum, they developed agar culture media for *H. pylori* growth (18, 22). Our results demonstrated that heat-inactivated fresh human serum could also preserve bacteria from freezing stress. This preservation was as high as with DMSO- and PEG-supplemented stock cultures with the values of 8 and 7 mean log CFU/ml for bacterial recovery at

-20°C and -80°C, respectively. The mechanism by which serum could protect bacteria has been mentioned by Albertson et al. Bovine serum albumin could reduce the toxic effects of fatty acids which are inhibitory substances for *H. pylori*. The absorption of cholesterol from serum as a growth factor for *H. pylori* might be another mechanism. Furthermore, the higher MICs of some chemical agents seen in serum-supplemented media could explain the reduction in bactericidal activity by serum (1).

Shahamat et al. suggested that the high concentration of serum in stock cultures containing glycerol may increase protection and prevent the destruction of internal structures of the bacteria due to ice crystal formation during the freezing process (21). Only control group A with BHI broth failed to yield any apparent colony counts; however, Kitsos et al. found that even Brucella broth alone could support some recovery of *H. pylori* at -80°C (14). This may be due to the type of bacterial strain, stock medium and culture medium, as well as the dilutions used for colony counts.

El-Shewy et al. showed that high-molecular weight compounds such as glycerol and DMSO had a more destructive effect on rat islet cell integrity and function than low-molecular ones such as PEG (7). Our study revealed that DMSO and PEG were also good cryoprotective agents, and we observed that the PEG-supplemented stock media were less effective than media with glycerol or DMSO in supporting the viability of *H. pylori*.

We also observed that the addition of 0.3 M sucrose to stock media resulted an increase in viability or culturability of *H. pylori* from stock cultures. Sato et al. showed that the addition of glucose to a liquid culture medium increased the culturability of *H. pylori* from a long-term culture. The highest culturability was obtained using liquid culture medium supplemented with 300 mM glucose. Glucose could prevent bacteria from changing from the proliferating spiral form to non culturable coccoid form (20). Our study showed a higher recovery of *H. pylori* from glycerol+sucrose-supplemented stock cultures than from glycerol alone. The sucrose used in our study might have the same protective mechanism in maintaining bacterial culturability from stock cultures. Another possibility was that the addition of sucrose at an optimal concentration might have altered osmotic pressure and caused dehydration of *H. pylori*, thereby reducing damage due to less ice crystal formation.

Albertson et al. showed that the addition of a carbon source such as glucose had no effect on the growth rate of *H. pylori* but that the viability of the organisms in the culture medium was retained after prolonged incubation. This slower conversion of bacteria to the non-culturable form could depend on the oxidative metabolism of *H. pylori* (1). Indeed, Hossain et al. demonstrated the protective effects of sucrose alone at concentrations of 100-300 mM in the survival of frozen human sperm (11).

Since we added only 0.3 M sucrose and since the results were not statistically significant, we suggest further investigation of various sucrose concentrations for improving the viability of *H. pylori* in stock cultures. In this study, we found that sucrose was effective in the cryopreservation of *H. pylori*. Its combination with BHI, glycerol and human serum resulted in a higher survival or culturability of *H. pylori* compared to other conventional stock media such as BHI with glycerol, although the difference was not statistically significant. Furthermore, the use of human serum in the culture and maintenance of *H. pylori* may be preferred, instead of bovine serum in which bovine spongiform encephalopathy (BSE) or other infections can be of serious concern (24).

In conclusion, our study showed that *H. pylori* type strain NCTC 11637 could be better preserved at -80°C than -20°C. The best stock medium that supported viability or culturability of bacteria was BHI+glycerol+human serum and BHI+glycerol +sucrose+human serum, where the latter gave a higher recovery rate. However, the difference was not found to be statistically significant. Our study also revealed that fresh human serum was a good, as well as more convenient, supplement for the growth and maintenance of *H. pylori* with less contamination.

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