Selected cryopreservatives for long term storage of *Helicobacter pylori* at low temperatures

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Abstract

To meet the need for information on cryopreservation, a study was done on 32 *Helicobacter pylori* strains, comparing different cryopreservative media. Sheep blood, horse blood, horse serum with and without glycerol, and mineral oil media were used for long term storage of *H pylori* at −70°C or in liquid nitrogen. Procedures were developed which permitted recovery of 87·5% of the strains included in the study after they had been stored for 24 months. Of those strains stored for more than three years, 60% were recovered. It is concluded that most strains of *H pylori* can be stored for up to one year or longer, under refrigeration, at −70°C or in liquid nitrogen.

Helicobacter pylori has an important role in the pathogenesis of chronic active gastritis type B and peptic ulceration in the upper gastrointestinal tract.[1] Studies of this micro-organism worldwide depend on the availability of pure, stable cultures of *H pylori*, especially those from intestinal biopsy specimens taken during clinical investigations. Such strains must be preserved for long intervals, using reliable and reproducible methods to avoid phenotypic or genetic variation or loss. Freeze-drying of *Helicobacter pylori* cannot be relied on to produce good recovery after subculture on solid media.[2] Furthermore, not every laboratory can afford equipment to freeze-dry *H pylori* directly from the liquid state.[3] Several freezing methods for long term storage of *H pylori*, using different cryoprotectants, have been described,[2-8] but a standard method for the preservation of *H pylori* is not available. We therefore undertook a study to compare cryoprotectants and evaluate critical technical steps in the cryopreservation of *H pylori*.

Methods

A total of 32 *H pylori* strains (table 1) were obtained from the National Collection of Type Cultures (NCTC). Before cryopreservation, cultures were grown on freshly prepared blood agar base No 2 (Oxoid), modified with 8% defibrinated horse blood, for 72 hours under microaerobic, humidified conditions at 37°C. *Helicobacter pylori* cells were harvested by scraping growth from the solid medium with a sterile spatula and resuspending the cells directly in cryoprotective media. The harvested cells were adjusted to concentrations of 10^9 to 10^{10} viable spiralic (non-coccoid) *H pylori* per ml in cryoprotective media. Enumeration was by acridine orange direct counting (AODC).[9] The cryopreservatives tested were defibrinated horse blood, defibrinated sheep blood, and horse serum (Remel, Lenexa, Kansas). The media tested consisted of one of these with and without 10% glycerol. Five strains were also tested further by the addition of 20 µl of sterile mineral oil to another series of phials with horse blood. Cryophials containing 0·5 ml of the bacterial suspension in the cryopreservative solutions were frozen immediately after inoculation at −70°C and a duplicate set was placed in liquid nitrogen. At established intervals, the frozen *H pylori* samples were thawed at room temperature. At recovery, the total number of cells was determined by AODC and recovery was measured by culturing samples on freshly prepared blood agar base No 2 (Oxoid) modified with 8% defibrinated horse blood. The recovered bacteria were confirmed as *H pylori* by epifluorescence microscopy and Gram stain. Urease, catalase, and oxidase tests were also performed.

Results and discussion

To establish a reliable procedure for cryopreservation of *H pylori* which can be used routinely in most microbiology laboratories,

Table 1 Strains of H pylori tested in this study

Source	Collection Number
NCTC	31155
NCTC	RSB6
NCTC	31604
NCTC	11219
NCTC	20200
NCTC	12954
NCTC	33097
NCTC	A-314
NCTC	33098
NCTC	12648
NCTC	60191
NCTC	60190
NCTC	TX30A
NCTC	95E
NCTC	11639
NCTC	11637
NCTC	26695
NCTC	26694
NCTC	7546
NCTC	11916
NCTC	48608
NCTC	52815
NCTC	43526
NCTC	189
NCTC	111
NCTC	99
NCTC	107
NCTC	53
NCTC	61
NCTC	110
NCTC	186
NCTC	115

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Table 2 Recovery of Helicobacter pylori from horse blood after storage for 24 months at −70°C

No of strains tested	Length of time held in storage	Efficiency of recovery	Percentage of recovered cells
8	24 months	++++*	25
15		+++	47
2		++	6
3		+	9
4		−	13

*Maximal cells recovered = ++++, good growth = +++, moderate growth = ++, weak growth = + and no growth = −.

conditions necessary for successful preservation of *H pylori* grown on solid media and using different cryopreservatives were evaluated. Horse blood and sheep blood, with and without glycerol, gave comparable results, with recovery of up to 60–80% of the original inoculum for most of the strains included in the study after storage for 24 months or longer. Recovery from horse serum was poor in some cases, or unsuccessful. Five of the strains of *H pylori*, randomly selected and frozen in horse blood with 20 µl sterile mineral oil, were not recovered at all.

Recovery success for 32 of the strains tested, calculated from number of colonies on blood agar plates after incubation for three days, showed that 28 of the 32 (87·5%) strains could be recovered after storage for 24 months at −70°C (table 2). Strain differences in the success of recovery were observed. Eight (25%) strains yielded full recovery, 15 (47%) showed good recovery, two (6%) moderate recovery, and three (9%) poor recovery after storage for 24 months (table 2). Of 32 strains tested, four (13%) could not be recovered at all. Thus some strains are more susceptible to storage conditions, regardless of the cryopreservative used.

Further analysis of the results showed that 15 of the 32 strains stored at −70°C in horse blood for 44 months could be cultured and nine of 15 (60%) strains could be recovered fully. On the other hand, two (13%) strains produced only a few colonies and four (27%) strains did not grow at all. As storage time increases, a larger number of strains lose the ability to grow on blood agar plates. A single cycle of thawing and freezing, for strains at the same storage temperature, did not reduce recovery significantly for most strains included in the study. However, when cultures were thawed and refrozen at a higher temperature (−20°C), most of the strains did not grow on blood agar plates under the same conditions as described above. Previous reports indicate that *H pylori* cannot be recovered even after one month if cell suspensions are transferred directly from plates to glycerol or glycerol plus fetal bovine serum.[3] The results obtained suggest that transferring *H pylori* directly from solid medium into blood will result, in most cases, in the bacteria being preserved for up to 44 months. However, storage in horse serum alone was not sufficient to preserve *H pylori* for the same period of time. According to published data, *H pylori* can be stored in Brucella broth in liquid nitrogen, but the length of the storage time was not reported nor was the efficiency of recovery.[8] Westblom *et al* recovered *H pylori* from horse blood stored at −70°C for six months. In the study reported here we recultured organisms after storage for 24 and 44 months at −70°C, with most of the cells viable and able to be cultured without difficulty.

In conclusion, criteria recommended for preservation and long term storage of *H pylori* are as follows. The initial inoculum should be larger than 5×10^9 cells/ml, and at least 90% of the cells should be vegetative, spiral-shaped cells when stained directly by AODC. Underlying this recommendation is our observation that coccoid forms readily arise in *H pylori* cultures when microenvironmental conditions are unfavourable for growth and the coccoid cells cannot be recultured easily, if at all, on solid media, using standard bacteriological culture methods (personal observations).[10] A storage temperature of −70°C is sufficient to provide a shelf-life of two years or more. Addition of glycerol to avoid ice crystal formation during the freezing process and, consequently, destruction of internal structures of the bacteria, will be less of a problem if the medium contains a high concentration of serum. Strain differences in response to these conditions, despite their being optimal for frozen storage, can be expected.

In any case, the conditions suggested above should permit successful frozen storage for most strains of *H pylori*, an important consideration when doing research on this fascinating, but often difficult (to culture) bacterium.

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