

The preservation of gonococci in liquid nitrogen

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SYNOPSIS Gonococci suspended in 1% proteose peptone containing 8% glycerol can be snap frozen in liquid nitrogen without detectable loss on thawing. This recovery rate has allowed the use of frozen organisms as the starting inoculum for the bulk growth of gonococci and to preserve gonococci both in urethral pus and after primary subculture for use in studies on gonococcal virulence.

The method described for freezing urethral pus on charcoal swabs should make it possible to transport infected specimens from areas lacking adequate laboratory facilities.

The long-term storage of standard strains of *Neisseria gonorrhoeae* is conveniently achieved by freeze drying but the recovery rates are poor, usually less than 5% (Brookes and Hedén, 1966). This selection pressure, together with the need for further subculture before use, are serious disadvantages in the study of gonococcal virulence. Techniques of storage at low temperatures can give survivals approaching 100% with other species of bacteria (Smith, 1961); such recovery rates would be ideal for maintaining the antigenically unstable gonococcus. Brookes and Hedén (1966), using a Linde controlled-rate freezer and storage in liquid nitrogen, obtained survivals of 20 to 30% with freshly isolated strains of *N. gonorrhoeae*. We report a simple technique of snap freezing in liquid nitrogen giving 100% recoveries with subcultured gonococci and an adaptation of the method for the preservation of urethral exudates.

Methods

ORGANISMS

Urethral exudates were obtained from men with acute gonorrhoea. Of the subcultured gonococci used, strains 1, 2, 3, and 4 were of colony types I or II (Kellogg, Peacock, Deacon, Brown, and Pirkle, 1963) while strains 5 and 6 were of colony types III or IV.

CULTURE

Urethral pus was cultured on Difco G.C. medium base containing dextrose 4 mg/ml, glutamine 100 µg/ml, ferric nitrate 10 µg/ml, and co-carboxylase 0.2 µg/ml (White and Kellogg, 1965). Viable counts were made in duplicate on plates of the same medium

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using automatic pipettes to deliver 10 µl aliquots from serial tenfold dilutions in 1% proteose peptone no. 3 (Difco). A triangular wire loop was used to spread the 10 µl volumes over the agar surface. All plates were incubated at 37°C in 5% CO₂ for 48 hours.

FREEZING TECHNIQUES

Subcultured gonococci were suspended in 1% proteose peptone containing different concentrations of either glycerol or dimethylsulphoxide (Kocka and Bretz, 1969) as protective agents. Duplicate 0.1 ml volumes containing about 10⁷ gonococci were dropped directly into liquid nitrogen contained in disposable polythene beakers. The frozen pellets were stored in screw-capped plastic ampoules (Sterilin Ltd) in a liquid nitrogen refrigerator.

Urethral pus was suspended in 8% glycerol peptone and 0.1 ml volumes were frozen either immediately after incubation at 37°C for 15 minutes or after homogenization using a Teflon grinder (Pierce, Dubos, and Schaefer, 1953). A further 0.1 ml of exudate was incubated at 37°C for 15 min in 8% glycerol peptone containing 5% saponin. Pellets were thawed rapidly by dropping into 0.9 ml of 1% proteose peptone maintained at 37°C in a water bath. In both sets of experiments the results of duplicate viable counts on each of two frozen samples were averaged and expressed as a percentage of the starting inoculum.

In a further series of experiments urethral exudates were collected on charcoal-impregnated swabs which were broken off into ampoules containing 0.4 ml of 8% glycerol peptone. After remaining at room temperature for periods of one to three hours, the swabs were cultured and then frozen by immersing the ampoules in liquid nitrogen.

Strain No.	Suspending Medium						
	Proteose Peptone	8% Glycerol Peptone	16% Glycerol Peptone	24% Glycerol Peptone	6% DMSO Peptone	16% DMSO Peptone	24% DMSO Peptone
1	13	98	58	76	37	56	15
2	50	98	100	115	98	87	23
3	18	132	112	98	48	21	16
4	27	63	76	72	57	29	27
5	25	140	100	135	56	56	20
6	22	115	107	105	66	49	13
Mean	26	107	92	100	60	49	19

Table I Effect of different concentrations of glycerol and dimethylsulphoxide (DMSO) on the survival of six strains of *Neisseria gonorrhoeae* frozen in liquid nitrogen¹

¹Recovery is expressed as a percentage of the original inoculum.

Homogenized in 8% Glycerol Peptone	Suspended in 8% Glycerol Peptone	Incubated 15 Min at 37°C in 8% Glycerol Peptone	Incubated 15 Min at 37°C in 8% Glycerol Peptone Containing 5% Saponin
102	33	59	40
105	36	40	36
150	36	66	66
121	40	86	97
185	15	138	62
Mean 133	32	79	60

Table II Effect of different processing methods before freezing on percentage recovery of gonococci frozen in urethral pus

Results

When some 10^7 viable units of subcultured gonococci were suspended in 0.1 ml of proteose peptone and added to liquid nitrogen only 26% of the organisms survived the freezing and thawing (Table I). The recovery rate was markedly improved by adding protective agents to the proteose peptone. When glycerol was used as the protective agent the average viable count after freezing often exceeded the inoculum count, presumably because clumps of gonococci were broken up during freezing and thawing. There was no significant difference between the number of gonococci recovered after freezing in 8, 16, or 24% glycerol. However, the number of gonococci recovered after freezing in 8% glycerol peptone was significantly higher than the number recovered from 6% dimethylsulphoxide peptone ($t = 2.975$, $df = 5$, $0.05 > P < 0.02$). At high concentrations dimethylsulphoxide appeared to be toxic because fewer gonococci were recovered after freezing in 24% dimethylsulphoxide than after freezing in proteose peptone alone.

The results of experiments on the preservation of gonococci in urethral exudates are given in Table II. When urethral pus was homogenized in 8% glycerol

peptone there was no loss of gonococci on freezing. In contrast, when the exudate was added to 8% glycerol peptone and immediately dropped into liquid nitrogen only 32% of the gonococci survived freezing. The loss can be explained by the failure of the protective agent to penetrate into urethral pus because survival increased to 79% when the exudate was incubated for 15 min at 37°C in 8% glycerol peptone. The addition of 5% saponin, which will rupture polymorphs without damaging gonococci (Watt, 1970), did not increase the percentage survival on freezing.

Gonococci were recovered from all of 20 specimens of urethral pus collected on charcoal-impregnated swabs and cultured after freezing and storing in liquid nitrogen for seven to 10 days.

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References

- Brookes, R., and Hedén, C. G. (1966). The preservation of *N. gonorrhoeae* by controlled-rate freezing followed by storage in liquid nitrogen. *Biotech. Bioeng.*, 7, 315-317.
- Kellogg, D. S., Peacock, W. L. Jr., Deacon, W. E., Brown, L., and Pirkle, C. I. (1963). *Neisseria gonorrhoeae*. I. Virulence genetically linked to clonal variation. *J. Bact.*, 85, 1274-1279.
- Kocka, F. E., and Bretz, W. H. (1969). Survival of *Escherichia coli* after storage in various frozen menstrua. *Antonie v. Leeuwenhoek*, 35, 65-76.
- Pierce, C. H., Dubos, R. J., and Schaefer, W. B. (1953). Multiplication and survival of tubercle bacilli in the organs of mice. *J. exp. Med.*, 97, 189-206.
- Smith, A. U. (1961). *Biological Effects of Freezing and Supercooling*, pp. 74-93, Arnold, London.
- Watt, P. J. (1970). The fate of gonococci in polymorphonuclear leucocytes. *J. med. Microbiol.*, 3, 501-509.
- White, L. A., and Kellogg, D. S. (1965). *Neisseria gonorrhoeae* identification in direct smears by a fluorescent antibody-counterstain method. *Appl. Microbiol.*, 13, 171-174.