

Preservation of *Neisseria gonorrhoeae* by the gelatin-disc method

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SUMMARY Studies of *Neisseria gonorrhoeae* are difficult to perform because of the organism's poor survival *in vitro*. To solve this problem we tried to preserve the organism by a gelatin-disc method. The rate of survival and changes of variations in some biochemical properties of eight strains of *N. gonorrhoeae* were followed for three years. These studies proved that preservation was satisfactory with only a 1/10 reduction of the living cells. Another trial showed that the organism survived for over six months after being frozen at -20°C . The colonial types, agglutination against red cells from rabbit and guinea pig, and antibiotic susceptibility to penicillin, chloramphenicol, tetracycline, kanamycin, and streptomycin did not change after three years' preservation.

Introduction

Kellogg *et al.* (1963, 1968) demonstrated a close correlation between colonial type and pathogenicity of *Neisseria gonorrhoeae*. Later Swanson *et al.* (1971) detected pili in pathogenic T1 and T2 colonies. Correlation between pathogenicity and colonial types and pili has also been reported by several investigators (Thongthai and Sawyer, 1973; Punsalang and Sawyer, 1973; Bumgarner and Finkelstein, 1973). Difficulties have been experienced in such studies, however, because the organism can only survive for a short time and mutation readily occurs with successive transfer of the subculture with loss of pathogenicity.

The most effective method for preserving *N. gonorrhoeae* now in common use is lyophilisation. Its application for a large number of strains, however, is complex and impossible in a small laboratory. We, therefore, attempted and successfully improved the preservation method by using desiccated gelatin, which was originally developed and effectively applied to the species of *Enterobacteriaceae* by Stamp (1947).

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Material and methods

MEDIA

Transgrow medium (Martin and Lester, 1971) for primary isolation and Difco GC medium base with 2% defined supplement (GCB-2DS) (Kellogg *et al.*, 1963) for confirmation of colonial-type and cell count were used.

GONOCOCCAL STRAIN AND COLONIAL TYPE

Eight fresh isolates of *N. gonorrhoeae* isolated in our laboratory in 1973-74 were used; strains 48-14 and 48-17 were isolated in 1973 from women with acute cervicitis. Strains 49-2, 49-13, 49-48, 49-52, and 49-60 were all isolated from men with acute urethritis and strain 49-8 was isolated from a woman with chronic cervicitis. The last six strains were isolated in 1974. They were identified by Gram staining, oxidase reaction, and carbohydrate fermentation. The colonial type was determined according to the definition of Kellogg *et al.* (1963) with the colony grown on GCB-2DS medium.

REAGENTS

Reagents for preparing gelatin-disc were prepared as follows:

- (1) Distilled water solution of Bacto dextrose (5%) with Bacto skim milk (3%) and de-colouring carbon (0.6%, Norit, Extra, NV

Norit-Vereeniging) was sterilised at 110°C for 10 minutes and stored in a refrigerator (solution A).

- (2) Distilled water solution of sodium L-ascorbate (0.5%, Wako) was sterilised by filtration through a millipore filter and stored at -20°C (solution B).
- (3) Distilled water solution of Bacto gelatin (20%) was sterilised at 121°C for 15 minutes and stored in a refrigerator (solution C).

As an antioxidant L-ascorbic acid, instead of sodium L-ascorbate, was also used for comparison, but the results were not promising.

INSTRUMENTS AND MATERIALS FOR PREPARING DISCS

Circular filter papers 7 cm in diameter (Toyo no. 2) were soaked in melted solid paraffin (melting point 56–58°C) (Wako) heated at 140°C for five minutes over a gas burner, removed with sterile forceps, and placed individually in a sterilised Petri dish, which was used as the receiver for droplets of cell suspension.

A glass desiccator with 500 g silica gel was used for drying; a glass cylinder with a wide opening containing 200 to 300 g of phosphorus pentoxide (P_2O_5) was placed in the desiccator.

A sterile capillary pipette giving a 0.03-ml droplet was used for dropping the cell suspension.

A 5-ml small vial with a wide opening, containing silica gel, was closed with a cotton plug and used for the storage of the desiccated gelatin discs containing the organisms.

PREPARATION OF GELATIN DISC

Each of the eight strains was inoculated on to two to three GCB-2DS agar plates and incubated at 35°C for 18 to 20 hours in a candle extinction jar. The organisms were collected to prepare a homogeneous suspension of 10^{10} cells/ml of solution A previously warmed to 35°C and stirred for 30 seconds with a vibrator. To this suspension were added similarly warmed 0.2 ml of solution B and 1 ml of solution C, which had been previously dissolved by heating and kept at 35°C; the suspension was stirred again for 30 seconds. The mixed suspension thus prepared was delivered dropwise with the capillary pipette on to the paraffin-soaked filter paper in the Petri dish. This Petri dish, with its cover slightly open, was placed in the desiccator and dried at room temperature under reduced atmospheric pressure (20 mm Hg [2.7 kPa]) with a vacuum pump. After four to six hours the hydrated portion near the surface of the P_2O_5 was removed to help further dehydration. It was then left overnight under a reduced pressure of 10 mm

Hg (1.35 kPa). After 24 hours the droplet had completely dried and changed into a thin and flat circular gelatin disc, which either came off the filter paper spontaneously or was lifted off with forceps. The discs of each strain were collected and put in the small vial, which was then tightly sealed and stored at -20°C.

VIABLE CELL COUNT

The quantitative cell count was performed on strains 48-14 and 48-17 immediately before and after drying and every month of each year thereafter. In addition, a pool of five discs from each strain was put into a small test tube, dissolved, and resuspended with 1 ml of heart infusion broth (Eiken) by heating at 35°C. After a series of tenfold dilutions of this original suspension had been prepared, each 0.1 ml of the dilution was streaked on a GC-2DS agar plate and then incubated at 35°C in a candle extinction jar for 24 hours. The number of colony-forming units (cfu) was thus counted, giving the number of viable cells per disc. In addition, six strains (49-2, 49-8, 49-13, 49-48, 49-52, and 49-60), were subjected to a qualitative study—that is, one disc from each strain was dissolved in 0.2 ml of heart infusion broth, one loopful of which was streaked on to a GCB-2DS agar and incubated at 35°C for 24 to 48 hours to determine colony-forming capacity.

STORAGE TEMPERATURE

To examine the effect of temperature on the survival of cells embedded in the gelatin disc, discs from the same batch were stored at different temperatures (that is, at 30°C, 5°C, and -20°C) and the change in cfu was followed with estimations at weekly intervals for six weeks and finally at 24 weeks.

ANTIBIOTIC SUSCEPTIBILITY TESTING

The method of Martin *et al.* (1970) was used for testing antibiotic susceptibility to penicillin, chloramphenicol, tetracycline, kanamycin, and streptomycin.

HAEMAGGLUTINATION REACTION

Red cells from rabbit and guinea pig were used according to the method of Punsalang and Sawyer (1973).

Results

All the eight strains stored survived three years' preservation. The decline of viable cells of strains 48-14 and 48-17 are shown in Figure 1. Despite a

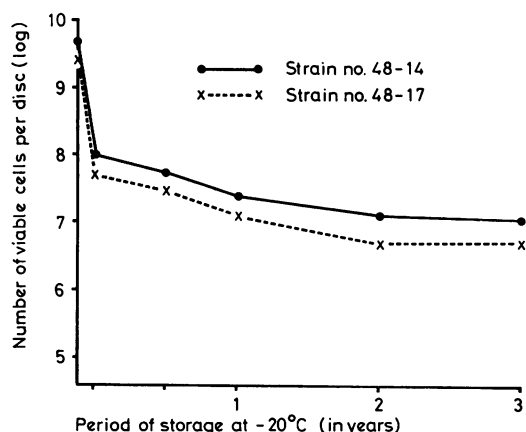


Fig. 1 Survival of *Neisseria gonorrhoeae* in gelatin discs

rapid decline of viable cells up to 10^{-2} of the initial suspension immediately after desiccation, the decline was less thereafter and dropped within the range of 10^{-1} of the initial suspension. The decline of viable cells, therefore, throughout the three-year preservation period was within the order of 10^{-3} of the initial suspension.

As shown in Table 1, colonial types were also maintained well after three years' preservation with only a slight decrease in frequency of appearance of the types 1 and 2 of some strains.

Tests of the effect of temperature on the viability of the cells embedded in gelatin disc showed that they were least affected when stored at -20°C even after six months, whereas no colony was formed after five to six weeks when stored at 30°C . The decline of viable cells after six months was within the order of 10^{-3} at -20°C and 10^{-4} at 5°C .

As regards the effect of antioxidants added to the mixed suspension, the decrease in viable cell count

Table 1 Percentage of colonial types of original and recovered cultures

Strain no.	% of colonial types			
	Original culture		Recovered culture (after 3 years)	
	Type 1-2	Type 3-4	Type 1-2	Type 3-4
48-14	10	90	10	90
48-17	10	90	10	90
49-2	90	10	90	10
49-8	90	10	80	20
49-13	90	10	90	10
49-48	90	10	70	30
49-52	90	10	80	20
49-60	90	10	90	10

immediately after freezing was less with sodium L-ascorbate than with L-ascorbic acid—that is, the former decrease was 10^{-2} whereas the latter was 10^{-3} of the initial suspension. The decrease while freezing was also less in the case of sodium L-ascorbate, the minimum effect being with the addition of 0.05 ml/ml giving a final concentration of 0.5%, and it was, therefore, appropriate for use. These effects are shown in Figure 2.

As shown in Table 2, no change was observed in the antibiotic susceptibility of any strains which were either highly sensitive or relatively resistant to penicillin or sensitive or resistant to streptomycin. They all agreed within a one-step difference of the gradation of minimum inhibitory concentration (MIC) after three years' preservation. Similarly, no change was observed in the susceptibility to chloramphenicol, tetracycline, and kanamycin.

No changes were seen in haemagglutination reaction when blood cells of either rabbit or guinea pig were used.

Comment and conclusions

Lyophilisation has been regarded as the most effective method for preserving strains of *N. gonorrhoeae*. Convenient methods have also been reported by Pospisil and Kabátová (1969), Sparling (1966), Thomas *et al.* (1973), Ofek *et al.* (1974),

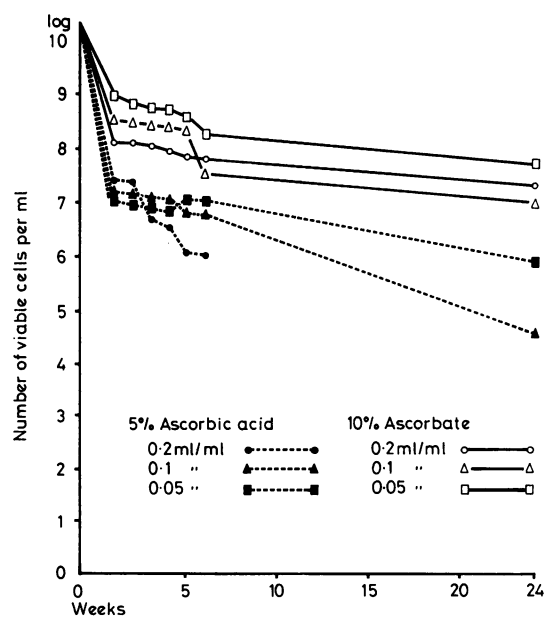


Fig. 2 Effect of antioxidants (sodium L-ascorbate and L-ascorbic acid) on survival of *Neisseria gonorrhoeae* during 24 weeks of preservation by freezing at -20°C

Table 2 Drug susceptibility of strains of *Neisseria gonorrhoeae* before and after storage for three years

Strain no.	Year	Drug susceptibility (MIC)				
		PC ($\mu\text{g/ml}$)	SM ($\mu\text{g/ml}$)	CP ($\mu\text{g/ml}$)	TC ($\mu\text{g/ml}$)	KM ($\mu\text{g/ml}$)
48-14	1974	0.03	6.3	4	1	8
	1977	0.02	12.5	4	1	4
48-17	1974	1	≥ 200	2	1	8
	1977	0.05	≥ 200	2	1	4
49-2	1974	2	≥ 200	8	4	16
	1977	1	≥ 200	8	4	16
49-8	1974	0.02	6.3	4	1	8
	1977	0.03	6.3	4	1	8
49-13	1974	1	≥ 200	4	2	8
	1977	1	≥ 200	4	4	16
49-48	1974	1	≥ 200	8	4	8
	1977	1	≥ 200	8	4	8
49-52	1974	1	≥ 200	2	0.5	8
	1977	1	≥ 200	2	1	8
49-60	1974	1	≥ 200	8	2	8
	1977	2	≥ 200	8	2	16

PC penicillin, SM streptomycin, CP chloramphenicol, TC tetracycline, KM kanamycin

and Apicella (1974). These lyophilisation or freezing methods, however, require expensive instruments and considerable space is occupied by the ampoules or tubes, which limits the number of strains to be stored.

In order to preserve strains of *N. gonorrhoeae* we attempted to apply and modify Stamp's (1947) classic simple method of preserving bacteria embedded in dried gelatin. For this purpose we used a lyophilisation medium, which we have routinely used for *Vibrio cholerae* strains, to which gelatin was added. The experiment resulted in success and *N. gonorrhoeae* was stored and kept alive by our gelatin-disc method for over three years. The properties which the organisms possessed at the time of fresh isolation were retained. Gelatin-mixed bacterial suspensions used for preparing the gelatin discs could also be stored in a viable state without desiccation by being frozen at -20°C for over six months.

Since our method is simple and requires neither expensive instruments nor large storage space, it is applicable in a small laboratory. The preservation of numerous strains under the same conditions in a compact state with many but small discs enables us to repeat our experiment and makes a long-term experiment feasible under the same conditions. Since the organism can also survive in gelatin discs at room temperature for several weeks, the method is simple and convenient for transportation.

When gelatin discs are prepared, the following should be observed:

- (1) Large amounts of a young culture incubated for 18 to 20 hours should be used.
- (2) A dense cell suspension, such as 10^{10} cells per ml, should be prepared.
- (3) Desiccation should be completed within a short time. The hydrated portion of P_2O_5 should, therefore, be removed and replaced with fresh P_2O_5 during the drying process.
- (4) Once desiccation is complete, the discs should be stored in a small vial with silica gel—that is, under dehydrated conditions at 5°C or -20°C .

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