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Survival of *Chlamydia trachomatis* in different transport media and at different temperatures: Diagnostic implications

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SUMMARY We compared the survival of a laboratory strain of *Chlamydia trachomatis* serovar L-2 in different media and at different temperatures (room temperature, 4°C, and −70°C). At these temperatures the best storage medium was 2SP (0·2 mol/l sucrose in 0·02 mol/l phosphate buffer supplemented with 10% fetal calf serum). We used material obtained from patients to study the sensitivity of the culture method as a function of sample storage time and temperature. Compared with results on direct inoculation, material stored in 2SP for 48 hours gave 11% fewer positive cultures at 4°C and 14% fewer at room temperature. Of samples which gave negative results on direct inoculation, 4% were positive after storage at 4°C for 48 hours and 2% after storage at −70°C for a week. As expected, the number of inclusion forming units in the original material proved to be important for the percentage of positive cultures among the stored samples.

Introduction

Culture of the organism from patient material on a suitable cell line is still the most important method of diagnosing chlamydial infection. Many studies have aimed at improvement of the culture method. The sampling of material[1,2] and the cell line[3,4] have all been subject to investigation. Few data are available, however, on the survival of *C trachomatis* in the most widely used transport medium, 2SP (0·2 mol/l sucrose in 0·02 mol/l phosphate buffer), and the diagnostic implications of survival rates. For such studies it is important to use patient material that, unlike clean laboratory strains, is rich in many substances and cells (such as leucocytes, anti-chlamydial antibodies, bacteria, and sperm constituents) which can influence the growth of chlamydiae on the monolayer. Reeve found a 17% decrease in positive cultures after storage of the material at −70°C for one week,[5] whereas Ngeow observed no loss after freezing the material in liquid nitrogen.[6] We studied a) the survival of a laboratory strain of *C trachomatis,* serovar L-2, in three different media and b) how storage of patient material under different conditions influenced the sensitivity of the culture method.

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Materials and methods

CULTURE PROCEDURE

A 100 µl sample of material to be tested for the presence of chlamydiae was inoculated on an HeLa 229 monolayer formed on a round coverslip in a flat bottomed tube. Except when the inoculum was the laboratory strain L-2, the monolayer was washed twice with 30 µg/ml diethylaminoethanol (DEAE) dextran in Hank's balanced salt solution before inoculation. After centrifuging the inoculated monolayers at 3700 *g* for one hour we added 1 ml growth medium which consisted of Eagle's modified minimal essential medium which contained 5% fetal calf serum, 4·5 mg/ml glucose, 25 U/ml nystatin, 25 µg/ml vancomycin and 25 µg/ml streptomycin (called CMGA). After three days the monolayers were stained by Giemsa's method and evaluated with dark field illumination at 250 × magnification. The number of inclusions per 30 microscopic fields or per coverslip was counted, and counts were made in triplicate.

SURVIVAL OF *C TRACHOMATIS* SEROVAR L-2 IN THREE MEDIA AT THREE TEMPERATURES

A 100 µl sample of chlamydia suspension in SPG

(75 g sucrose, 0·52 g potassium phosphate, 1·22 g disodium acid phosphate, and 0·72 g L-glutamine, dissolved in water in a ratio of 1:1) was added to 0·9 ml of the test medium. The three media used were Earle's balanced salt solution, CMGA, and 2SP, and all contained 10% fetal calf serum. Portions of the medium containing chlamydiae were incubated at 4°C and at room temperature and frozen at −70°C. Samples were obtained at intervals and the number of inclusion forming units (ifu) in them was measured.

PROCESSING OF PATIENT MATERIAL

An aluminium swabstick (ENT swab, Boehringer, Mannheim, FRG) was used to obtain a urethral sample from men attending the outpatient clinic for sexually transmitted diseases (STD) of the Rotterdam University Hospital. The material was suspended in 2SP and divided into three portions; one was stored at 4°C, the second at −70°C, and of the third portion a fraction was inoculated immediately while another fraction was stored at room temperature. After 24 and 48 hours the samples stored at 4°C and at room temperature were inoculated in triplicate. After one week the samples stored at −70°C were thawed and inoculated. The 2SP medium also contained 25 U/ml nystatin, 20 μg/ml vancomycin, and 20 μg/ml streptomycin.

TABLE I *Comparison of survival of C trachomatis serovar L-2 after suspension in three media at three temperatures for varying times*

Storage temperature and time	*Mean No (%) inclusions per 30 microscope fields (× 250 magnification) of three coverslips produced by 10^{-3} dilution of the suspensions on:*		
	CMGA	*EBS*	*2SP*
Room temperature			
4 hours	263 (100)	358 (100)	396 (100)
8 hours	213 (81)	344 (96)	293 (74)
25 hours	33 (13)	41 (11)	179 (45)
50 hours	3 (<1)	3 (<1)	22 (6)
4°C			
4 hours	396 (100)	446 (100)	571 (100)
50 hours	10 (3)	15 (3)	30 (>5)*
80 hours	8 (2)	15 (3)	30 (>5)*
120 hours	1 (1)	1 (1)	30 (>5)*
−70°C			
2 days	223 (100)	205 (100)	284 (100)
6 days	16 (7)	16 (8)	57 (20)†
1 week	12 (5)	13 (6)	50 (18)†
2 weeks	26 (12)	22 (11)	62 (22)†

CMGA = Eagle's modified minimal essential medium; EBS = Earle's balanced salt solution; 2SP = 0·2 mol/l sucrose in 0·02 mol/l phosphate buffer.
*At 50, 80, and 120 hours >100 inclusion were counted in a 10^{-1} dilution on 2SP.
†2SP proved to be significantly ($p<0·05$) (Student's *t* test) superior to the other media.

Results

Our comparison of survival in different culture media showed that chlamydiae survived best in 2SP at all temperatures studied. Table I lists the number of inclusions obtained by inoculating a 10^{-3} dilution of the suspensions in phosphate buffered saline (PBS). Undiluted 2SP suspensions produced 158 and 96 inclusions after storage for 150 and 174 hours respectively at room temperature. Clinical conditions were probably best mimicked by diluted suspensions. In general these showed substantial reduction of inclusions after storage for 25 hours at room temperature, 50 hours at 4°C, and six days at −70°C.

TABLE II *Number of positive cultures obtained from patient material after immediate inoculation compared with results after storage*

Storage temperature and time	*No of positive cultures after:*	
	Immediate inoculation	*Storage (%)*
Room temperature		
24 hours	46	39 (85)
48 hours	44*	38 (86)
4°C		
24 hours	46	41 (89)
48 hours	45*	40 (89)
−70°C		
1 week	44*	39 (89)

*Some samples were not tested under all storage conditions.

Table II shows the results of our investigation into the effect of storing material from patients on chlamydial survival. Storing reduced the number of positive cultures by 11-15% depending on the storage temperature. Storing patient material also unmistakably reduced the number of inclusions per coverslip. Table III shows that this reduction depended on storage times and temperatures and amounted to 40-70% of the number of inclusions formed after inoculation of the material immediately after

TABLE III *Effect on number of inclusions by storing patient material at three temperatures for different times*

Storage temperature	*Percentage of inclusions on immediate inoculation after storage for:*		
	24 hrs	*48 hrs*	*1 week*
Room temperature	50	30	
4°C	60	60	
−70°C			55

Significant (Student's *t* test; $p<0·05$) difference between number of inclusions after 48 hours at 4°C and one week at −70°C, and between 48 hours at 4°C and 48 hours at room temperature.

sampling. Eleven positive specimens became negative under one or several storage conditions and averaged seven inclusions per coverslip, while 27 remained positive under all storage conditions and averaged 98 inclusions per coverslip. Table IV shows that in a group of patient samples which gave negative results after immediate inoculation, 4% (7 of 178) gave positive results after storage for 48 hours at 4°C, and 2% (4 of 162) after 1 week at −70°C.

TABLE IV *Number of cultures of patient material which were negative after immediate inoculation and positive after postponed inoculation*

No of negative cultures after immediate inoculation	*No (%) of positive cultures after:*	
	48 hrs at 4°C	*1 week at −70°C*
178	7 (4)	
162*		4 (2)

*No material was stored at −70°C from 16 patients.

Discussion

The experiments with our laboratory strain L-2 show that 2SP is a better storage and transport medium than Earle's balanced salt solution and CMGA. We do not know why survival is better in 2SP, but the fact that sucrose is cryoprotective may play a part.[7]

As might be expected, the sensitivity of the culture diminishes when the patient material is not immediately inoculated on the monolayer. The number of inclusions decreases noticeably, but the influence of this decrease on the culture is of course limited. After all the demonstration of only one inclusion is enough to give a positive result. A correlation exists, however, between the number of inclusion forming units in the initial material and the reversion of culture results after storage, with fewer inclusions increasing the risk of reversion. Apart from the difficulty of finding a single inclusion in a specimen, the degeneration of chlamydiae constitutes a real problem in this context.

Immediate freezing, the procedure routinely followed for patient material, produces a loss of 11% inclusions. When the unfrozen material is in transit for one or two days this loss increases to 25%. The loss is compensated for only partly by positive results in material found to be negative after immediate inoculation. A possible explanation for this finding lies in the intracellular localisation of chlamydiae. Freezing or storage can cause the eukaryotic host cells to break open. Brief sonication of the patient material immediately before inoculation would therefore seem to be rational. The disintegration of toxic material during storage might also play a part.[6]

Facilities for culturing chlamydiae are restricted to a few centres. Proper processing of the materials can therefore encounter logistical problems which unfavourably affect the sensitivity of the culture method. Ridgway inoculated patient material immediately and after transport to a central reference laboratory, which entailed loss of 22·6%.[8]

This study stresses the necessity to process patient material as quickly as possible. One has to deal with an unacceptable loss in sensitivity by transporting material for chlamydial culture, unless there are laboratory facilities to inoculate specimens directly.

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