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Viability Change of *Mycobacterium Tuberculosis* in the Sputum Specimens Stored at Different Temperatures with or without Preservatives

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—국문초록—

객담보관온도별 결핵균의 생활력 변화와 오염방지를 위한 방부제에 관한 연구

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보관온도 및 기간에 따른 객담내 인형결핵균의 생활력 변화와 객담오염 방지를 위한 방부제의 선택을 위해 도말염색표본에서 현미경으로 항산균을 검출할 수 있었던 41개의 객담으로 관찰한 결과를 보면 다음과 같다. 객담을 4℃에 보관할 경우 1주까지는 모두 배양검사에서 결핵균이 발육했으나, 2, 3, 4주에서는 각각 9.8%, 19.5% 및 26.8%의 객담이 배양에서 결핵균이 발육하지 않았다. 25℃에서는 1주에서도 19.5%의 객담이 배양음성이었고, 2, 3, 4주에서는 각각 36.6%, 70.7%, 90.2%가 배양음성이라 고온보관으로 균이 많이 사멸하는 것을 알 수 있었다. 25℃에서 객담을 1주이상 보관하면 오염율도 매우 높았다. 이와 같은 오염을 0.5% boric acid(BA), 5% trisodium phosphate(TP) 및 0.5% cetylpyridium chloride(CP)첨가로 막을 수 있으나, CP를 제외하고는 결핵균에 유독해 방부제로 적합하지 않았다. CP첨가 객담은 25℃이상의 여름철 기온에서 3, 4주간 방치해도 각각 61.0% 및 31.7%가 배양에서 결핵균이 발육하였다. 그런데 BA첨가 객담은 같은 기간에 각각 34.1% 및 4.9%만이 배양양성이라 균에 유독함을 알 수 있었다. 따라서 여름철과 같이 기온이 높을 때 객담을 배양할 수 있는 검사실까지 운송하는데 2주이상 걸리는 상황에서는 CP가 유용한 방부제로 이용될 수 있다고 본다.

Key Words: *M. tuberculosis*, sputum, temperature, preservatives.

INTRODUCTION

Culture examination of sputume or other clinical specimens is necessary for the accurate diagnosis and treatment control of tuberculosis and thus it must be organized in the laboratory networks of the national tuberculosis programme. The small laboratories in the peripheral health institutes are usually lack of culture facility and a skilled technician and so, if sputum cultures are required, the specimens must be transported to the larger laboratory. In this case the long delayed arrival of the specimens are often received and, if the specimens have been left exposed to high ambient temperature as in summer, many of the specimens are spoiled causing high contamination rate and a significant loss of viability of tubercle bacilli. This situation urged a number of investigators to study on the preservatives possibly useful to protect specimens (sputums) from contamination (1-4). Some have been found useful for the preservation of the sputum specimens during transport.

In this study, change of cultivable bacilli of *M. tuberculosis* present in the sputum specimens upon storage at the different temperatures with or without preservatives has been investigated and a preservative selected has been evaluated for a possible use in the routine laboratory services.

MATERIALS AND METHODS

1. Sputum specimens.

Sputum specimens were collected from the patients who visited the Korean Institute of Tuberculosis Cinic (KITC) for the first time or who were under the treatment. Altogether 41 smear positive sputum specimens were selected for the present study.

2. Sputum treatment.

Each sputum specimen was divided into 5 parts (each part, approximately 3 ml) and they were treated separately, for example two were stored at 4℃ and 25℃ respectively and three with three different preservatives. Sputum was mixed with an equal volume of 1% boric acid (BA), 1% cetylpyridium chloride (CP), or 10% trisodium phosphate (TP) and stored at room temperature (25-30℃). These specimens wee processed for culture of tubercle bacilli after 0, 3, 7, 14, 21, and 28 days of storage.

3. Sputum cultures

Sputums stored at 4℃ or 25℃ were decontaminated with 4% sodium hydroxide and then inoculated onto the two slopes of acid-buffered Ogawa medium. The BA treated sputum was mixed with an equal volume of 4% sodium hydroxide and CP or TP treated specimens with an equal volume of sterile distilled water and then inoculated onto the media. Left-over preservatives treated specimens were diluted with sterile water and centrifuged at 1,500g for 20 minutes. The sediments were planted onto the media. The cul-

Table 1. Effect of temperature in storage of sputum specimens on culture of tubercle bacilli

Temperature of storage	No. sputums studied	Duration of storage (days)					
		0	3	7	14	21	28
4°C	41	41 (100.0)	39 (95.1)	41 (100.0)	37 (90.2)	33 (80.5)	30 (73.2)
	Media cont.		1 (1.2)				1 (1.2)
25°C	41	41 (100.0)	37 (90.2)	33 (80.5)	26 (63.4)	12 (29.3)	4 (9.8)
	Media cont.			5 (6.1)	14 (17.1)	14 (17.1)	9 (11.0)

*( ) =%, ** Media cont. = media contaminated.

Table 2. Viability change of tubercle bacilli in sputums stored at different temperatures

Duration of storage (days)	4°C			25°C		
	No decrease	Considerable decrease	Great decrease	No decrease	Considerable decrease	Great decrease
0	41 (100.0)			41 (100.0)		
3	39 (95.1)	2 (4.9)		34 (82.9)	2 (4.9)	5 (12.2)
7	35 (85.4)	4 (9.8)	2 (4.9)	22 (53.7)	10 (24.4)	9 (22.0)
14	24 (58.6)	9 (22.0)	8 (19.5)	11 (26.8)	4 (9.8)	26 (63.4)
21	14 (34.1)	14 (34.1)	13 (31.7)	3 (7.3)	3 (7.3)	35 (85.4)
28	13 (31.7)	6 (14.6)	22 (53.7)	0 ( )	3 (7.3)	38 (92.7)

* ( ) = %

** No decrease = no significant decrease in number of colonies grown on culture when compared with that of the same specimens prior to storage.

*** Considerable decrease = decreases of colony number from +++ (numerous) or ++++ (confluent) to + (20-100) or from ++ (100-200) to 5-19 or from + to < 5.

**** Great decrease = decrease of colony number from +++ or ++++ to < 20 or 0 or from ++ to < 5 or 0 or from + or < 20 to 0.

Table 3. Effect of preservatives on the protection of sputums (41) from contamination and on the viability of tubercle bacilli.

Preservatives	Culture methods & contamination	Duration of storage (days)					
		0	3	7	14	21	28
0.5% boric acid (BA)	Direct	41 (100.0)	38 (92.7)	38 (92.7)	31 (75.6)	13 (31.7)	5 (12.6)
	Conta.	0	0	0	0	0	2 (2.4)
	Concent.	41 (100.0)	36 (38.7)	37 (90.2)	14 (34.1)	2 (4.9)	
	Conta.	0	0	0	0	0	2 (2.4)
0.5% cetylpyridium chloride (CP)	Direct	41 (100.0)	38 (92.7)	33 (80.5)	26 (63.4)	16 (39.0)	11 (26.8)
	Conta.	0	0	0	4	2	4 (4.9)
	Concent.	41 (100.0)	35 (85.4)	31 (75.6)	30 (73.2)	25 (61.0)	13 (31.7)
	Conta.	0	0	0	0	0	2 (2.4)
5% trisodium phosphate (TP)	Direct	41 (100.0)	30 (73.2)	7 (17.1)	0	0	0
	Conta.	0	1 (1.2)	1 (1.2)	0	1 (1.2)	0
	Concent.	41 (100.0)	33 (80.5)	13 (31.7)	2 (4.9)	0	0
	Conta.	0	0	1 (1.2)	0	&	2 (2.4)

* ( ) = %

** Conta. = contamination, Concent. = concentration

tures were incubated at 37℃ for up to 8 weeks before discarding as negative.

RESULTS

As seen in table 1, it was clear that viability of tubercle bacillin could be preserved much better at 4℃ than at 25℃ as the culture positives were 80.5%, 63.4%, 29.3% and 9.8% respectively after 1, 2, 3, and 4 weeks of storage at 25℃ while they were 100.0% 90.2%, 80.5%, and 73.2% after respective durations of storage at 4℃. Contamination was negligible among the sputums stored at 4℃, but it was considerably high among the specimens stored at 25℃ for more than one week. As the loss of viability was inevitable even at 4℃ if sputums were stored for long perid of time, sputum cultures should be performed preferably within 7 days from the collection. A great loss of viable counts was observed in 19.5% and 53.7% of sputums for 2 and 4 weeks of storage even at 4℃. Situation was much worse at 25℃ showing great decrease in 22.0%, 63.4%, 85.4%, and 92.7% for 1, 2, 3, and 4 weeks of storage. One week storage did not significantly decrease viable counts of tubercle bacilli in 85.4% of sputums if the specimens were kept at 4℃, but remained unchanged in only 53.7% of the sputum specimens if they were stored at 25℃. Beyond one week of storage, number of specimens shown no significant loss of viable count were drastically decreased even at 4℃. Therefore it may require some measure to protect the viability of tubercle bacilli and the sputums from contamination if the sputum culture delayed beyond one week at 25℃ or higher. All three preservatives studied protected sputums from contamination fairly well, however the loss of culture positivity was unacceptably high in sputums added 5% trisodium phosphate while the other two preservatives did not reduce the number of culture positives to a great extent up to two weeks storage at room temperature (25-30℃). When storage of sputums prolonged to 3 to 4 weeks, the loss of culture positives was significantly higher among sputums treated by 0.5% boric acid than among sputums treated by 0.5% cetylpyridium chloride as seen in table 3. Culture positives were 34.1% and 4.9% among BA treated sputums on 3rd and 4th week of storage, while 61.0% and 31.7% of positive

cultures were found among CP treated sputums on the same periods of storage. And concentration of CP treated specimens yielded apparently more positives than the direct inoculation method. However the preservation of sputums without loss of viability could be achieved better by low (4℃) temperature storage than by cetylpyridium chloride treatment at 25℃.

DISCUSSION

It is of course desirable to process sptutum culture as soon as possible from the time of collection. However many of the sputum specimens are exposed considerably high temperature (25-30℃) for long period of time particularly in summer season if they have to be transposted to the larger laboratory where culture facility is available, as is often the case in many developing countries. Under such circumstance, high contamination rate and significant loss of viability of tubercle bacilli is inevitable. Paramasivan, *et al.* (5) reported that storage of sputums at tropical temperature (25-30℃) reduced from 87.8% of positive cultures prior to storage to 68.3%, 22.5%, 12.5%, and 0% after 1, 2, 3, and 4 weeks respectively. And contamination was increased from 7.3% prior to storage to 30.0% after two weeks. Similar finding was obtained in this study, showing that positive cultures have been reduced from 100.0% prior to storage to 80.5% by one week storage of sputums at 25℃ and 63.4% by two weeks, 29.3% by three weeks, and 9.8% by four weeks of storage.

Therefore, if it is inevitable to transport sputums to another laboratory in high ambient temperature taking more than one week as is often the case in many deveoping countries, there must be some measure to protect sputums from contamination and viability of tubercle bacilli. Kudoh and Kudoh (1) tested 0.5% boric acid to prevent contamination of sputums and they found that it inhibited contamination to some extent but decreased viability of tubercle bacilli, thus use of BA as preservative had no advantage at all. Our study confirmed their finiding. There were no apparent differences in yields of culture positives between BA contained and free sputums although much low contamination was encountered in BA treated specimens. Cetylpyridium chloride, however, was much less harmful to the viability of tubercle bacilli than BA and yet prevented contamination fairly well if CP treated specimen was centrifuged prior to inoculation on the medium. A number of investigators (2-4) also reported that CP decontaminated sputums without reducing viability of tubercle bacilli. Tazir, *et.al.* (4) suggested that further digestion of CP treated specimens should be helpful to obtain positive cultures from specimens containing small number of bacilli, otherwise bacilli may be decanted due to their poor sedimentation on centrifugation. Trisodium phosphate (5%) was worst of all tested.

Still sputums could be preserved best by storing at 4℃, thus efforts should be made store the sputum specimens in a refrigerator if avilable and transport them as quickly as possible.

SUMMARY

The viability of tubercle bacilli in the sputum specimens has been investigated after different periods of storage at diffeent temperatures and in the presence of different preservatives. No loss of culture positives was observed for one week storage at 4℃, but 9.8% 19.5%C, and 26.8% of sputums failed to yield positives on 2, 3, and 4 weeks of storage respectively. At 25℃ even one week storage made 19.5% of sputums fail to yield positive culture and 2, 3, and 4 weeks of storage made 36.6% 70.7%, and 90.2% of sputums fail to yield positive culture respectively. And contamination was unacceptably high beyond one week of storage at 25℃. Contamination of sputum specimens could be protected fairly well by 0.5% boric acid, by 5% trisodium phosphate or by 0.5% cetylpyridium chloride, but, except CP, the former two had no advantage at all to protect viability of tubercle bacilli over the specimens without preservative. The CP was much less harmful to the viability of tubercle bacilli than BA, yielding 61.0% and 31.7% of culture positives on 3

and 4 weeks of storage in the presence of CP, while BA yielded 34.1% and 4.9% of positives on the same respective periods of storage. Therefore CP may be useful to preserve sputums if it takes more than 2 weeks to transport them at the temperature of over 25℃.

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