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Original Article

Evaluation of Swab in Maintaining Survival Efficiency according to the CLSI M40-A2 Standard

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Transporting clinical samples for microbiological testing requires a proper transport medium that guarantees the survival of microorganisms. Therefore, the aim of the study was to determine the ability of Amies Transport Medium (ATM) to maintain the viability of microorganisms in clinical specimens and its suitability as a transport medium for microbiological testing. This study evaluated the performance of swab provided by KS Co., Ltd. for three groups of bacteria comprising aerobic and facultative anaerobic bacteria, anaerobic bacteria, and fastidious bacteria, according to the Clinical and Laboratory Standard Institute (CLSI) 8.11.2. The ATM stability test was conducted by dividing the medium into two groups based on the product expiration date of use. All tested media, A and B (the date of manufacture and expiration date are different) showed ≥5 CFU, and there was no significant difference in the result values of Category A and Category B with different serial numbers for each test. The results of this experiment when cross-checked with the guidelines suggest that ATM is a suitable transport medium for microbiological testing, as it maintains the viability of microorganisms and is suitable for overgrowth trials. In addition, compared to the number of CFUs at the origin, the number of CFUs did not increase by more than 1 log after storage. These results have important implications for the development of transport media that can guarantee the survival of microorganisms in clinical specimens.

Key Words: Transport medium, CLSI, Viability, Overgrowth, CFU, Bacteria

INTRODUCTION

Although various devices are available to transport specimens, based on the type of specimen and culture environment of the bacteria being transported, a common method of transport involves a plastic tube containing a bacterial medium and a sterile disposable swab.

Microorganism recovery from clinical specimens requires proper specimen collection and transportation (Tyrrell et al.,

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2016). Maintaining the viability and relative proportions of all microorganisms in the clinical specimen is essential during transportation (Wayne, 2010). A swab collection and transport device are frequently used in hospitals and other healthcare settings to obtain clinical specimens. It is crucial to collect, transport, and inoculate samples for microbiological testing (Drake et al., 2005; Gizzie and Adukwu, 2016).

If testing cannot be performed promptly after collection, the sample should be stored in a suitable environment or moved to a location where microbiological testing is possible. However, it may take several to 24 h from sample collection to the microbial laboratory; if the transportation is delayed, microbial isolation may fail depending on the state of the preserved sample.

The Amies Transport Medium (ATM, KS Co., Ltd. Naju, Korea) is an *in vitro* diagnostic medical device used for maintaining and preserving the viability of bacteria while transporting samples collected from patients to the laboratory for bacterial testing. Therefore, in this study, we aim to use ATM to evaluate the viability of aerobic, facultative anaerobic, anaerobic, and fastidious bacteria according to the Clinical and Laboratory Standard Institute (CLSI), M40-A2 guidelines (Perry, 1997; Farhat et al., 2001; Hindiyeh et al., 2001; Van Horn et al., 2008; Namekar et al., 2013). In addition, we conducted a stability test for ATM to determine whether the bacterial culture remains active until the expiration date of the transport media. There are two methods of bacterial inoculation. First, the initial inoculation method is to collect the bacteria by the Medschenker's nylon flocked swab and inoculate it immediately (5 min) at 20~25℃. Another method is inoculation after storage, in which the bacteria are smeared on the MedSchenker's Flocked swab, stored for 24 or 48 h at 4~8℃, and then inoculated into the medium (Wayne, 2010).

MATERIAL AND METHOD

The test was performed using a single colony after culturing the standard strain on a media plate for 24~48 h using Medschenker's nylon flocked swabs. Absorbance was measured at a wavelength of 600 nm according to the guidelines of a 0.5 McFarland turbidity standard in the range 0.08~1 (approximately cell density 1.5×10^8 CFUs/mL). Qualitative criteria are as follows: the inoculum concentration was prepared according to the guidelines of the CLSI 8.12.1., and three bacterial groups (aerobic bacteria, anaerobic bacteria, and picky bacteria) were evaluated. There are two methods of bacterial inoculation. First, the initial inoculation method is to collect the bacteria by the Medschenker's nylon flocked swab and inoculate it immediately (5 min) at 20~25℃. Another method is inoculation after storage, in which the bacteria are smeared on MedSchenker's Flocked swab, stored for 24 or 48 hr at 48℃, and then inoculated into the medium. The experiment was repeated thrice in the same manner. For the ATM stability tests until the expiration date, we used different serial numbers for each test (Table 1).

Cell line

This study was conducted in the Biosafety Level 2 laboratory in the Department of Clinical Pathology, Catholic University of Pusan. Test strains are the standard strains presented in the (CLSI) guidelines. This study was conducted by purchasing standard strains from Korean Collection for Type Cultures (KCTC, Daejeon, Korea) and American Type Culture Collection (ATCC, Virginia, USA) (Table 2).

Study isolates

According to the CLSI 8.11.2., three groups of bacteria (aerobic and facultative anaerobic bacteria, anaerobic bacteria, and fastidious bacteria) were evaluated. This collection of three groups of bacteria test isolates consisted of four aerobic and facultative anaerobic bacteria reference isolates (*Pseudomonas aeruginosa* KCTC 22074, *Streptococcus pyogenes* ATCC 19615, *Streptococcus pneumoniae* ATCC 6305, *Haemophilus influenzae* ATCC 10211), five anaerobic bacteria reference isolates (*Bacteroides fragilis* KCTC 5013, *Peptostreptococcus anaerobius* ATCC 27337, *Fusobacterium nucleatum* KCTC 2640, *Cutibacterium acnes* ATCC 6919, *Prevotella melaninogenica* KCTC 5457) and one fastidious bacteria reference isolates (*Neisseria gonorrhoeae* ATCC 43069) that are difficult to culture were used.

Table 1. To confirm the qualitative criteria, the detailed number of tests is shown in the table below

Serial number (Lot No.)	Features	Organism	Dilution ratio	Bacterial inoculation	Number of quantity
Category A Solid type with charcoal (KLA21G1) Liquid type (KLB21G2)	**Category A** Manufacture date 2021. 07. 01.	*P. aeruginosa* *S. pyogenes* *S. pneumoniae* *H. influenzae* *B. fragilis* *P. anaerobius* *F. nucleatum* *C. acnes* *P. melaninogenica* *N. gonorrhoeae*	10^3	Initial inoculation immediately: 3 Inoculation after storage: 3	270
			10^4	Initial inoculation immediately: 3 inoculation after storage: 3	
			10^5	Initial inoculation immediately: 3 Inoculation after storage: 3	
Category B Solid type with charcoal (KLA22D1) Liquid type (KLB22D2)	**Category B** Manufacture date 2022. 04. 01.	*P. aeruginosa* *S. pyogenes* *S. pneumoniae* *H. influenzae* *B. fragilis* *P. anaerobius* *F. nucleatum* *C. acnes* *P. melaninogenica* *N. gonorrhoeae*	10^3	Initial inoculation immediately: 3 Inoculation after storage: 3	270
			10^4	Initial inoculation immediately: 3 Inoculation after storage: 3	
			10^5	Initial inoculation immediately: 3 Inoculation after storage: 3	

Table 2. The standard strains used in the experiment

Organism	Strain	Inoculum (CFU)	Medium	Incubation temperature (℃)	Incubation atmosphere
Aerobic, facultative anaerobic bacteria					
Pseudomonas aeruginosa	KCTC 22074	$10^3 \sim 10^5$	TSA + 5% sheep blood	35±2	Aerobic
Streptococcus pyogenes	ATCC 19615	$10^3 \sim 10^5$	TSA + 5% sheep blood	35±2	5% CO_2
Streptococcus pneumoniae	ATCC 6305	$10^3 \sim 10^5$	TSA + 5% sheep blood	35±2	5% CO_2
Haemophilus influenzae	ATCC 10211	$10^3 \sim 10^5$	Chocolate agar	35±2	5% CO_2
Anaerobic bacteria					
Bacteroides fragilis	KCTC 5013	$10^3 \sim 10^5$	BHI agar	35±2	Anaerobic
Peptostreptococcus anaerobius	ATCC 27337	$10^3 \sim 10^5$	BHI agar	35±2	Anaerobic
Fusobacterium nucleatum	KCTC 2640	$10^3 \sim 10^5$	BHI agar	35±2	Anaerobic
Cutibacterium acnes	ATCC 6919	$10^3 \sim 10^5$	BHI agar	35±2	Anaerobic
Prevotella melaninogenica	KCTC 5457	$10^3 \sim 10^5$	BHI agar	35±2	Anaerobic
Facultative anaerobic bacteria					
Neisseria gonorrhoeae	ATCC 43069	$10^3 \sim 10^5$	Chocolate agar	35±2	5% CO_2

CLSI M40-A2 roll-plate method

The experiment was conducted using the roll-plate method. Microorganisms grown for 24~48 hr were diluted with 0.85% physiological saline (pH 6.8~7.2) to approximately 1.5×10^8 CFU (equivalent to 0.5 McFarland turbidity standard) inoculation solution concentration. The inoculated solution is subjected to 1:10 serial dilution four times and adjusted such that the final concentration falls within 1.5×10^7~1.5×10^4 CFUs/mL. For each microbial group to be

evaluated, 100 μL is added to 96-well for each diluent of 1.5×10^4, 1.5×10^5, and 1.5×10^6 and inoculated using a cotton swab. The final inoculations absorbed by the swab were 1.5×10^3, 1.5×10^4, and 1.5×10^5 CFUs/mL. Two types of bacterial swabs were used to inoculate into different types of ATM of A (liquid type) and B (solid type with charcoal) with varying dates of expiration. First, the initial inoculation method is to collect the bacteria by the Medschenker's nylon flocked swab and inoculate it immediately (5 min) at 20~25℃. Another method is inoculation after storage, in which the bacteria are smeared on Med-Schenker's Flocked swab, stored for 24 or 48 hr at 4~8℃, and then inoculated into the medium. The experiment was repeated thrice in the same manner.

Viability test and check for overgrowth

After incubation, a viability test was performed by counting the CFUs and calculating the average. Each medium was evaluated according to the inoculation time and dilution factor. The final CFUs were calculated as the average of the CFUs of the three culture plates. The plate of origin medium closest to 250 CFUs was used to qualify for compliance in a viability test. The viability test was performed with an appropriate dilution factor and storage time. If the result is

Table 3. Viability and overgrowth tests after storage at refrigerated and room temperature (The Liquid type)

Organism	Type	Recovery at concentration of [a]					
		10^5 CFU/mL at (h)		10^4 CFU/mL at (h)		10^3 CFU/mL at (h)	
		5 min	24 hr, 48 hr	5 min	24 hr, 48 hr	5 min	24 hr, 48 hr
		RT	4℃	RT	4℃	RT	4℃
Aerobic, facultative anaerobic bacteria							
P. aeruginosa	A	725	655	142	120	12	13
	B	815	648	155	135	15	14
S. pyogenes	A	302	316	35	32	4	3
	B	319	267	42	30	4	3
S. pneumoniae	A	608	758	74	90	9	9
	B	749	971	90	107	10	11
H. influenzae	A	1389	1311	213	174	20	19
	B	1230	1281	183	171	17	17
Anaerobic bacteria							
B. fragilis	A	1429	1412	313	313	29	28
	B	1442	1358	321	314	30	27
P. anaerobius	A	1513	1453	226	216	24	23
	B	1421	1435	212	216	24	23
F. nucleatum	A	1212	1264	244	244	21	20
	B	1181	1103	241	205	20	18
C. acnes	A	1258	1170	288	253	30	28
	B	1241	1162	257	222	25	21
P. melaninogenica	A	1111	1419	180	204	20	22
	B	1375	1391	213	221	24	24
Fastidious bacteria							
N. gonorrhoeae	A	1227	956	251	228	29	27
	B	1060	869	216	211	27	29

[a]Average of triplicate tests in CFU/100 μL. RT, room (ambient) temperature
A and B the date of manufacture and expiration date are different

Table 4. Viability and overgrowth tests after storage at refrigerated and room temperature (The solid type with charcoal)

Organism	Type	Recovery at concentration of [a]					
		10^5 CFU/mL at (h)		10^4 CFU/mL at (h)		10^3 CFU/mL at (h)	
		5 min	24 hr, 48 hr	5 min	24 hr, 48 hr	5 min	24 hr, 48 hr
		RT	4℃	RT	4℃	RT	4℃
Aerobic, facultative anaerobic bacteria							
P. aeruginosa	A	907	819	160	157	14	13
	B	820	764	152	150	13	12
S. pyogenes	A	327	252	30	24	3	3
	B	350	273	44	26	4	2
S. pneumoniae	A	809	837	104	112	9	9
	B	937	854	122	115	10	9
H. influenzae	A	1525	1400	221	184	18	18
	B	1413	1418	180	196	20	19
Anaerobic bacteria							
B. fragilis	A	1545	1450	329	294	32	29
	B	1522	1360	322	296	31	28
P. anaerobius	A	1530	1521	247	240	26	24
	B	1490	1571	243	254	26	26
F. nucleatum	A	1282	1135	251	211	23	21
	B	1347	1251	286	234	25	22
C. acnes	A	1554	1447	327	309	31	31
	B	1508	1461	301	317	35	34
P. melaninogenica	A	1557	1677	253	252	23	22
	B	1353	1607	228	248	22	22
Fastidious bacteria							
N. gonorrhoeae	A	1062	974	218	187	27	29
	B	993	931	208	177	29	25

[a]Average of triplicate tests in CFU/100 μL. RT, room (ambient) temperature
A and B the date of manufacture and expiration date are different

≥ 5 CFUs, it indicates that it meets the criteria as suitable. The check for overgrowth is the average number of colonies in the origin plate that should be 5~50 CFUs to effectively perform the overgrowth experiment. For the results of an overgrowth test to be considered acceptable, the number of CFUs after storage should not increase by more than 1 log compared to the number of CFUs measured in the plate of origin. For example, if the plate of origin had 15 CFUs, it is considered acceptable if the overgrowth plate test has less than 150 CFUs after storage.

RESULTS

In this study, the CLSI M40-A2 roll plate method was used; two types of ATM were used for transport systems, viz., liquid type and solid type with charcoal. Bacteria used in the study were three groups of bacteria (aerobic and facultative anaerobic bacteria, anaerobic bacteria, and fastidious bacteria). A total of 10 bacteria were cultured in two media types with different manufacture dates. This process was repeated thrice (Table 2).

Recovery of Liquid type transport system

Of the two Amies Transport Medium (ATM) systems, the liquid-type transport system showed acceptable recovery from all ten isolates according to CLSI standards. Recoveries of the aerobic and facultative anaerobic bacteria after storage at room temperature and refrigeration are summarized in Table 3. The final CFUs were calculated as the average of the CFUs of the three plates. If the result is ≥ 5 CFUs, it indicates that it meets the criteria as suitable in a viability test. To consider it acceptable in the overgrowth test, the number of CFUs after storage should not increase by more than 1 log compared to the number of CFUs at the origin. As a result, ≥ 5 CFUs was found in all tested media, and accordingly all ten isolates showed acceptable recovery, and overgrowth did not increase by more than 1 log.

Recovery of Solid type with charcoal transport system

Of the two ATM systems, the solid type with the charcoal transport system showed acceptable recovery from all ten isolates according to CLSI standards. Recoveries of the anaerobic bacteria after storage at room temperature and refrigeration are summarized in Table 4. The final CFUs were calculated as the average of the CFUs of the three plates. If the result is ≥ 5 CFUs, it indicates that it meets the criteria as suitable in a viability test. To consider it acceptable in the overgrowth test, the number of CFUs after storage should not increase by more than 1 log compared to the number of CFUs at the origin. As a result, ≥ 5 CFUs was found in all tested media, and accordingly all five isolates showed acceptable recovery, and overgrowth did not increase by more than 1 log.

DISCUSSION

It is essential to collect, transport, and inoculate samples for microbiological testing. The CLSI M40-A2 provides standardized methods for both roll plate method and elution, to aid manufacturers and laboratories in determining the performance characteristics of swab transport devices. If testing cannot be performed promptly after collection, the sample should be stored in a suitable environment or moved to a location where microbiological testing is possible. However, it may take several to 24 h from sample collection to the microbial laboratory; if the transportation is delayed, microbial isolation may fail depending on the state of the preserved sample. Therefore, the transport medium must ensure the viability of microorganisms in clinical specimens, and the performance of the transport medium is very important for the accurate diagnosis of clinical trials.

The criteria set by the CLSI M40-A2 standard for the roll plate method is as follows: all samples stored at 4℃ and room temperature for viability compliance must yield ≥ 5 CFUs after the specified storage period. The collected samples were evaluated for the ability to maintain the viability of bacteria in the two types (liquid type and solid type with charcoal) of transport medium, which is the ATM provided by KS (Avolio and Camporese, 2015). Three groups of bacteria (aerobic and obligate anaerobic bacteria, anaerobic bacteria, and fastidious bacteria) were evaluated according to CLSI 8.11.2 (Rishmawi et al., 2007). For ATM stability tests until expiration, we used Category A and B with different serial numbers for each test.

As a result, ≥ 5 CFU was found in the tested media A and B (the date of manufacture and expiration date are different). According to the guidelines, the results of this experiment were proved to be suitable for the viability test. In addition, compared to the number of CFUs at the origin, the number of CFUs did not increase by more than 1 log after storage. It has also proven to be suitable in overgrowth trials.

In conclusion, the ATM system is an acceptable swab transport system for aerobic, anaerobic, and picky bacteria. This system met CLSI acceptance criteria for all aerobic and anaerobic isolates when tested under refrigerated storage conditions. Therefore, it is considered that this result is suitable as a transport medium for transport to a place where microbiological testing is possible.

Compliance with ethical standards

This study was conducted by purchasing standard strains from KCTC (Daejeon, Korea) and ATCC (Virginia, USA). As such, no consent was required from study subjects.

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CONFLICT OF INTEREST

The authors declare that they have no commercial or financial relationships that could be perceived as a potential conflict of interest in conducting this research.

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