A Novel Technique of Aluminum Multiplier Slide Culture for Fungal Identification

Lalita Matthapan, B.Sc., Waranyoo Prasong, B.Sc., Charussri Leeyaphan, M.D., Sumanas Bunyaratavej, M.D., Kamonpan Lertrujiwanit, B.Sc.
Department of Dermatology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand.

ABSTRACT
Objective: Single slide culture technique (SSC) is the standard method of preparing fungal colonies for species identification. As contamination was frequently found, restarting the process wasted time and delayed diagnosis for patients. Aluminum slide culture (ASC) is the novel method, which aims to increase more fungal colonies in same time. Even though, some colonies showed contamination, there would be others left to use for identification. This study aimed to evaluate contaminate rate and time consumed in ASC technique to identify mold fungi compared to SSC.

Methods: ASC and SSC were used to identify 50 fungal colonies comprising 3 species of dermatophytes including *Trichophyton mentagrophytes*, *T. rubrum*, *Microsporum canis*, and 2 species of non-dermatophytes including *Neoscytalidium dimidiatum* and *Fusarium solani* which are common causative molds for superficial fungal skin and nail infection. Fungal colonies were microscopically examined every 5 days. Time to species identification and contamination rate were recorded and analyzed.

Results: This study demonstrated that ASC had equal efficacy in fungal identification with SSC. Median time from inoculation to fungal identification in ASC technique was 15 days which was significantly less than in SSC technique (18 days, \( p \)-value ≤ 0.05). At 15 days after inoculation, contamination rate revealed 2% in ASC whereas in SSC it was significantly higher at 16%.

Conclusion: ASC technique is an effective alternative method to prepare fungal colonies for identification. This study demonstrated that ASC uses less time to identify mold fungi and revealed lower contamination rate than SSC technique.

Keywords: Slide culture technique; fungal identification; morphological identification (Siriraj Med J 2018;70: 438-441)

INTRODUCTION
Superficial fungal infections such as skin and nail infections were caused by dermatophytes (DMPs) and nondermatophytes (NDMs). Prevalence of DMPs onychomycosis reported by previous studies was 75.9% in which *Trichophyton mentagrophytes* was the leading agent. Of patients who had NDMs onychomycosis, 17.3% were *Neoscytalidium dimidiatum* and 6.8% were *Fusarium* spp.1 Clinical characteristics were not distinguishable between DMPs and NDMs except patients with NDMs onychomycosis showed absence of fungal glabrous skin infection in areas other than the feet. NDMs onychomycosis were considered recalcitrant to systemic treatment. Causative fungal identification was essential for making decision on patients’ treatments. Early treatment could be initiated as soon as causative fungus was identified.1,2 Fungi culture remains the gold standard method to identify fungal species.3 Specimens were collected from skin or subungual hyperkeratosis lesions and cultured. If fungal colonies were detected after specimen culture,
single slide culture (SSC) was performed for specific fungal detection. Fungus would take around 1-3 weeks to produce spores and colonies. Macroscopic and microscopic examinations were used to classify fungal species. SSC could provide well-preserved morphology of the fungus and the slide from this technique could be kept as the semi-permanent slide for studying later. On the other hand, Tease slide and scotch-tape technique could be used to prepare colony for microscopic examination, but these techniques could disturb colony formation which resulted in difficulty to identify fungal species. Contamination was the main problem for SSC. If there was fungal contamination in SSC after culture, the process had to restart which resulted in time and money wasted. Moreover, prolonged fungal identification may lead to delay in specific treatment for patients.

Aluminum slide culture (ASC) technique is the novel innovative method, which aims to solve contamination problems in SSC. Generally, SSC contained one set of slide and cover glass for one colony formation. Conversely, ASC had 4 sets of 4 cover glasses for four colony formations. If contamination was found in one colony in ASC, there were others left to use for fungal identification without restarting process. This study aimed to evaluate time consumed for fungal identification and contamination rate of ASC, compared with SSC. This study will demonstrate the efficacy of ASC which will be a promising method for colony culture in general mycology laboratories.

MATERIALS AND METHODS
Specimen preparation procedures
Specimens were collected from scale or sunungual hyperkeratosis lesions of patients with superficial fungal infections. The specimens were placed in sabourauds dextrose agar (SDA) with cyclohexamide and chloramphenicol and SDA with chloramphenicol and inoculated at 25-30°C and 30% humidification. Normally, fungus takes around 1-3 weeks to form the specific colonies. Five fungal species, common causative species for superficial skin and nail infections including *Trichophyton mentagrophytes, T. rubrum, Microsporum canis, Neoscytalidium dimidiatum* and *Fusarium solani* were used in this study. Each species were performed 10 times therefore in total 50 plates were performed for ASC and 50 plates for SSC techniques.

Single slide culture technique (SSC)
A sterile glass slide and v-shaped glass rod on the sterile petri plate was prepared as Fig 1A. SDA, block sized 1x1 cm³ was placed in the middle of the glass slide. Sterile needle was used to inoculate fungal colony to all 4 sides of SDA block and cover glass was put on the top of SDA block. Some sterile water was filled into the petri plate to prevent the drying of SDA block. The slide was incubated at 26°C.

Aluminum slide culture technique (ASC) 87
A sterile aluminum block with 4 cover glasses and 4 SDA blocks was placed on the sterile petri plate. Each SDA block sized 1x1 cm³ were placed in the middle of each cover glass (Fig 1B). Sterile needle was used to inoculate fungal colony to all 4 sides of every SDA block and cover glass was put on the top of every SDA block. Some sterile water was filled into the petri plate to prevent the drying of SDA block. The slide was incubated at 26°C.

Interpretation and analysis of the test
Growth and contamination of the fungal colonies were examined every 5 days under low power microscopic examination (10x). After 15 days of inoculation, cover glasses were removed from sterile plate, placed on to the glass slide and examined under lacto phenol cotton blue and under light microscope.

Measurement of time consuming for fungal identification
During inoculation period, if fungal contamination was detected in SSC technique, restart of SSC technique was required to identify species. On the other hand, in ASC technique, even if some specimens were contaminated, the others would be left to use to identify fungal species. Except for all blocks of ASC were contaminated, restart of ASC technique was not performed. Duration from
inoculation to fungal identification was collected and analyzed.

**Measurement of contamination rate**

Contamination was reported every 5 days if there was other fungal growth in SDA blocks in both techniques. Regarding ASC, if any one block was contaminated, contamination rate was reported immediately. Contamination rate and the day that contamination was found were collected and analyzed.

**Statistical analysis**

Mann-Whitney U test and Fisher’s exact test were used for analyses of time consumed to identify fungi species and contamination rate, $p \leq 0.05$ was considered as significant difference.

**RESULTS**

In total 50 plates of ASC and 50 plates of SSC were performed in this study. Fungal identification from ASC technique showed the same result with SSC techniques, which represented equal efficacy for both techniques. Median time from inoculation to fungal identification in ASC technique was 15 days while in SSC technique it was 18 days. Median time from inoculation to fungal identification in ASC technique was significantly less than in the SSC technique ($p$-value $\leq 0.05$). From the SSC technique, there were 2, 3 and 3 specimens had contamination and had to restart the whole process on day 5th, 10th and 15th after inoculation.

Rate of contamination was shown in Table 1. After 5 days of inoculation, contamination was found in 2 (4%) $T. \text{rubrum}$ slides in SSC and 1 (2%) $T. \text{rubrum}$ slide in ASC technique. On the 10th day, contamination was revealed in 5 (10%) slides in SSC technique comprising 2 $T. \text{rubrum}$ slides, 1 $T. \text{mentagrophytes}$ slide, 2 $M. \text{canis}$ slides; whereas there was only 1 (2%) $T. \text{rubrum}$ slide in ASC technique. On the 15th day, contamination was demonstrated in 8 (16%) slides in SSC including 2 $T. \text{rubrum}$ slides, 2 $T. \text{mentagrophytes}$ slides, 4 $M. \text{canis}$ slides which was significantly different from 1 (2%) $T.\text{rubrum}$ slide in ASC technique with $p$-value $=0.031$.

**DISCUSSION**

SSC technique was the standard method of preparing fungal colonies for species identification. ASC is the novel method, aiming to produce more fungal colonies in the same time. This study demonstrated that ASC had equal efficacy on fungal identification as SSC. However, median time from inoculation to fungal identification in ASC technique was significantly shorter than in SSC technique. At 15 days after inoculation, contamination rate revealed 2% in ASC whereas in SSC it was found to be significantly higher at 16%. This study demonstrated that ASC technique would be an effective alternative method to prepare fungal colonies for identification.

SSC technique was recommended by Lewis and Hopper in 1943 as the standard method of preparing fungal colonies for species identification with well-preserved morphology of the fungi. Each SSC technique provided one fungal colony to use for identification. In clinical practice at mycology laboratory, contamination was the main problem in SSC technique. Time and materials would be consumed more when the whole process required restart. Delayed fungal identification leads to difficulty in diagnosis and treatment. Modified culture slide technique was previously reported by Rosana et al., which placed the agar directly on the sterile plate and inoculated fungal colony at 4 sides of agar then cover glasses were put on the top surface of the agar. Another technique used inoculation of the mole fungi directly on the sterile petri plate and place the agar on top later. However, ASC was different from other modified techniques. ASC was invented as the new technique to increase slides with the aluminium block at the same inoculation time.

**TABLE 1.** The comparison of contamination rate in SSC and ASC technique.

<table>
<thead>
<tr>
<th>Day</th>
<th>Slide culture technique (N=50)</th>
<th>Aluminum slide culture technique (N=50)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 5</td>
<td>2 (4%)</td>
<td>1 (2%)</td>
<td>1.000</td>
</tr>
<tr>
<td>Day 10</td>
<td>5 (10%)</td>
<td>1 (2%)</td>
<td>0.204</td>
</tr>
<tr>
<td>Day 15</td>
<td>8 (16%)</td>
<td>1 (2%)</td>
<td>0.031*</td>
</tr>
</tbody>
</table>

*p-value <0.05 indicates statistical significance*
Generally, SSC technique provided one slide for fungal identification; while ASC technique could produce 4 slides in the same time. Therefore, ASC had greater chances of success in mold fungi identification, but used less storage space, so the cost benefit should be greater.

According to efficacy for fungal identification, ASC technique was equivalent to the standard technique; SSC technique. This confirmed that ASC technique could be an alternative technique to culture fungi to use for identification. Median time from inoculation to fungal identification in ASC technique was significantly less than the SSC technique (p-value ≤ 0.05) in this study. Due to 4 slides available for colony formation in ASC technique, even if one slide got contaminated, the others slides could be continuously used for fungal documentation. Rapid and early fungal identification could be obtained from ASC which were valuable for patients’ diagnosis and treatment. Cost reduction between ASC and SSC would need to be further elucidated.

DMPs and NDMs which were common causative agents for superficial skin and nail infections were studied in this report. Further study would be required to demonstrate efficacy for fungal identification in slow growing molds such as Magnaporthe grisea and Exophiala jeaneselmei causing eumycetoma, Pocilopora verrucosa, Fonsecaea compacta and Fonsecaea pedrosoi causing chromoblastomycosis, and Euphaisea spinifera causing phaeohyphomycosis. Due to slow growing molds, ASC is expected to significantly reduce time for fungal identification compared with SSC.

For the contamination rate, this study showed that ASC technique has significantly less contamination rate compared with SSC technique. In point of fact, ASC technique required to open the cover of petri plate for longer time than the SSC technique because inoculation had to be done for 4 SDA blocks in ASC, compared to only 1 SDA block in SSC. Formerly, contamination rate was expected to be higher in ASC due to prolonged opening of plate. However, this study did not show higher contamination rate in ASC, conversely this study reported that rates of contamination were the same on the 5 th and 10 th days and significantly lower in ASC on the 15 th day. All processes in this study were done by one experienced technician and inoculations in the 2 techniques were done at the same time. Manual error was minimized in this study, so the reason for lower contamination rate with ASC may need further study. Unfortunately, from our literature review, data regarding contamination rate from other modified culture techniques was limited to comparisons with ASC technique.

CONCLUSION
The ASC technique is a novel method of preparing fungal colonies for species identification. The quality of identification of mold fungi is equivalent compared with the SSC technique, but better in less contamination rate and less time consumed to identify mold fungi species.

ACKNOWLEDGMENTS
The authors would like to thank Assistant Professor Dr. Orawan Supapueng for her advice about statistical analysis and Department of Dermatology, Siriraj Hospital for support for the research and laboratory in this study.

REFERENCES