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Note

Impact of Spreading Time to Recovery Rate in Suitability Test of Solid Agar Media

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In these days, all agar media used for both pharmaceutical and industrial territories were required to meet performance criteria. There were recovery rates of assigned microorganisms as performance criteria in both pharmacopeia and ISO standards. However, in spreading plate method, there is no concrete spreading time even though it is shown only "as quickly as possible" in ISO standards. In this study, we verified the impact of spreading time in spreading plate method for the quality control of SCD (Soybean Casein Digest) agar plate. When 30s, 60s, and 120s of spreading time were compared using *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 6538, *Candida albicans* ATCC 10231, and *Aspergillus brasiliensis* ATCC 16404, respectively, there is no significant difference in recovery rates of all strains tested between 30s and 60s. However, recovery rates of *E. coli* and *P. aeruginosa* were decreased in 120s of spreading time. Our results demonstrated that spreading using plastic rod would be better to complete within 60s in spreading plate method since long spreading time had the impact to recovery rate of certain bacteria.

Key words : Media quality test / Spreading plate Method / Japanese pharmacopeia / ISO 11133.

For industrial use, the agar media have been widely used for both the enumeration and selective differentiation. In pharmaceutical territory, all media used are required to meet performance criteria of pharmacopeia. For ISO standard methods in food microbiology, all media used for food and animal feed also need to satisfy performance criteria of each ISO standard document as well as pharmacopeia. In these backgrounds, the performance criteria of agar media have gradually been rigidified. According to Japanese pharmacopeia, media used for both microbial limit test and sterilization test need 50-200% recovery rate of the reference count in assigned quality control strains as performance criteria (Ministry of Health, Labour and Welfare, 2016). In a similar fashion, ISO 11133 specifies that non-selective and selective media used for ISO standard need >70% and >50% recovery rate of assigned quality control strains as performance criteria, respectively (International Organization for Standardization, 2014).

In general, the procedure of spreading plate method is to spread inoculum uniformly on the surface of the solid agar plate using sterile hockey rod shaped spreader. However, in performance test for solid agar media, there is no mention for spreading time in the inoculation of quality control strains in both Japanese pharmacopeia and ISO 11133 even though quality control strains should be used within 2 hours in both standards. Further, both ISO 7218 as general rule, and ISO 4833-1 as surface plating technique show only "as quickly as possible" with respect to spreading time in spreading plate method even though it is shown only that surface of media can be dry for enough absorbance (International Organization for Standardization, 2007 and 2013). As just described, spreading time is very ambiguous factor in spreading plate method since the degree of dry of the

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surface of agar plate is different for individual agar plate.

Hence, it needs to verify acceptable period for spreading time in inoculation of quality control strains for the quality control of solid agar media. The aim of this study was to evaluate the impact of spreading time for inoculation in spreading plate method.

For tested strains, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 6538, *Candida albicans* ATCC 10231, and the spore of *Aspergillus brasiliensis* ATCC 16404 (American Type Culture Collection, Manassas, VA) were used in this study according to Japanese pharmacopeia. Test procedure was also according to Japanese pharmacopeia. In brief, bacterial strains were cultured on SCD agar plate (SCD: Soybean Casein Digest, Nippon Becton Dickinson Company, Ltd., Tokyo, Japan) at 35°C for 24h. *C. albicans* was cultured on Sabouraud dextrose agar (Nippon Becton Dickinson) at 25°C for 72h. The spore of *A. brasiliensis* was recovered after cultured on Sabouraud dextrose agar (Nippon Becton Dickinson) at 25°C for 72h. Each microorganism tested was suspended in phosphate buffered saline, pH 7.2 (PBS) and was then subjected to dilution to make ca. 100 CFU/0.1mL of microbial suspension. Each 0.1mL of bacterial suspension was inoculated onto 5 plates of each SCD agar (Nippon Becton Dickinson), Tryptic soy agar (Merck KGaA, Darmstadt, Germany), and Trypto-Soya agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), respectively. Inoculated test strains on each 5 solid agar media were spread using sterile plastic rod (Nissui Pharmaceutical Co., Ltd.) for 30s, 60s, and 120s, respectively. After bacterial strains were cultured at 35°C for 48h, the number of colonies was counted. For *C. albicans* and *A. brasiliensis*, the number of colonies was counted after cultured at 25°C for 72h. All statistical analyses were conducted by using Microsoft Excel 2019 at significant level of $P = 0.05$. The means and standard deviations (SD) of 5 replicates with respect to each spreading time were calculated. A one-way analysis of variance (ANOVA) was also performed to determine the differences among each spreading time.

Table 1 shows the result of recovered number of each tested strain using SCD agar. The mean ± SD of number of grown colony for each tested strain in 30s of spreading time were 73.0 ± 5.7 (*B. subtilis*), 78.6 ± 6.7 (*E. coli*), 117.4 ± 6.3 (*P. aeruginosa*), 90.8 ± 6.8 (*S. aureus*), 58.0 ± 3.6 (*C. albicans*), and 30.2 ± 5.0 (*A. brasiliensis*), respectively. For 60s of spreading time, the mean ± SDs of those were 74.8 ± 4.2 (*B. subtilis*), 74.8 ± 8.7 (*E. coli*), 117.0 ± 7.3 (*P. aeruginosa*), 94.8 ± 7.6 (*S. aureus*), 56.4 ± 2.5 (*C. albicans*), and 28.0 ± 3.7 (*A. brasiliensis*), respectively, and showed equivalent

TABLE 1. Summary of numbers of grown colony for each spreading time on SCD agar [a]

Strain tested [b]	*B. subtilis*			*E. coli*			*P. aeruginosa*			*S. aureus*			*C. albicans*			*A. brasiliensis*		
Spreading time	30s	60s	120s	30s	60s	120s	30s	60s	120s	30s	60s	120s	30s	60s	120s	30s	60s	120s
Mean of grown colony [c] (CFU / plate)	73.0	74.8	71.2	78.6	74.8	17.6	117.4	117.0	16.8	90.8	94.8	90.6	58.0	56.4	59.0	30.2	28.0	31.4
SD	5.7	4.2	4.9	6.7	8.7	7.1	6.3	7.3	6.3	6.8	7.6	10.7	3.6	2.5	5.1	5.0	3.7	4.7
p-value against result of 30s [d]	—	0.62	0.64	—	0.51	1.5×10^{-6}	—	0.94	1.6×10^{-8}	—	0.46	0.98	—	0.49	0.76	—	0.50	0.74

[a] SCD agar was manufactured by Nippon Becton Dickinson Company, Ltd.
[b] All bacterial stains tested were cultured at 35°C for 48h. *C. albicans* and *A. brasiliensis* were cultured at 35°C for 72h.
[c] Tests of each microbe were conducted at 5 replicates.
[d] ANOVA was performed at a significans level of 0.05. The *p* value <0.05 is a statistically significant difference between the two conditions,

TABLE 2. Summary of numbers of grown colony for each spreading time on Tryptic soy agar [a]

Strain tested [b] Spreading time	*B. subtilis*			*E. coli*			*P. aeruginosa*			*S. aureus*			*C. albicans*			*A. brasiliensis*		
	30s	60s	120s	30s	60s	120s	30s	60s	120s	30s	60s	120s	30s	60s	120s	30s	60s	120s
Mean of grown colon [c] (CFU/plate)	74.0	71.6	72.0	78.0	71.0	24.6	121.0	116.2	22.4	82.6	88.6	84.0	58.0	55.8	57.0	30.2	28.8	31.6
SD	3.2	4.8	4.6	4.8	6.7	7.4	6.2	5.8	8.5	3.8	8.7	7.2	3.7	5.4	4.2	2.8	3.3	3.9
p-value against result of 30s [d]	—	0.43	0.50	—	0.13	2.1×10^{-6}	—	0.29	6.8×10^{-8}	—	0.24	0.74	—	0.52	0.73	—	0.54	0.58

[a] Tryptic soy agar was manufactured by Merck KGaA.
[b] All bacterial stains tested were cultured at 35°C for 48h. *C. albicans* and *A. brasiliensis* were cultured at 35°C for 72h.
[c] Tests of each microbe were conducted at 5 replicates.
[d] ANOVA was performed at a significans level of 0.05. The p value<0.05 is a statistically significant difference between the two conditions,

TABLE 3. Summary of numbers of grown colony for each spreading time on Trypto-Soya agar [a]

Strain tested [b] Spreading time	*B. subtilis*			*E. coli*			*P. aeruginosa*			*S. aureus*			*C. albicans*			*A. brasiliensis*		
	30s	60s	120s	30s	60s	120s	30s	60s	120s	30s	60s	120s	30s	60s	120s	30s	60s	120s
Mean of grown colony [c] (CFU/plate)	73.6	71.2	70.0	75.2	72.2	5.6	123.2	122.2	19.2	74.2	81.0	76.6	52.6	54.4	53.2	30.4	30.8	31.0
SD	3.5	3.4	5.1	6.1	8.1	3.2	6.2	4.5	7.7	8.1	8.2	8.7	1.9	3.9	2.6	4.3	2.5	4.1
p-value against result of 30s [d]	—	0.35	0.28	—	0.57	3.9×10^{-8}	—	0.80	2.8×10^{-8}	—	0.27	0.70	—	0.43	0.72	—	0.88	0.85

[a] Trypto-Soya agar was manufactured by Nissui Pharmaceutical Co., Ltd.
[b] All bacterial stains tested were cultured at 35°C for 48h. *C. albicans* and *A. brasiliensis* were cultured at 35°C for 72h.
[c] Tests of each microbe were conducted at 5 replicates.

value with that of 30s. On the other hand, the mean ± SD of those for 120s of spreading time were 71.2 ± 4.9 (*B. subtilis*), 17.6 ± 7.1 (*E. coli*), 16.8 ± 6.3 (*P. aeruginosa*), 90.6 ± 10.7 (*S. aureus*), 59.0 ± 5.1 (*C. albicans*), and 31.4 ± 4.7 (*A. brasiliensis*), respectively. For 120s of spreading time, *B. subtilis*, *S. aureus*, *C. albicans*, and *A. brasiliensis* showed equivalent mean ± SD with that for both 30s and 60s. However, the mean ± SD of *E. coli* and *P. aeruginosa* for 120s of spreading time were dramatically decreased. As a result of statistical analysis (ANOVA), there was no significant difference in recovery rate of *B. subtilis*, *S. aureus*, *C. albicans*, and *A. brasiliensis* among 3 periods of spreading time. For *E. coli* and *P. aeruginosa*, recovery rate in 120s of spreading time had significant difference with that of 30s.

Table 2 and 3 show that the result of recovered number of each tested strain using Tryptic soy agar and Trypto-Soya agar, respectively. Both Tryptic soy agar and Trypto-Soya agar were also according to Japanese pharmacopeia as well as SCD agar with respect to formulation. In case of Tryptic soy agar, the mean ± SD of number of grown colony for each tested strain in 30s and 60s of spreading time were 74.0 ± 3.2 and 71.6 ± 4.8 (*B. subtilis*), 78.0 ± 4.8 and 71.0 ± 6.7 (*E. coli*), 121.0 ± 6.2 and 116.2 ± 5.8 (*P. aeruginosa*), 82.6 ± 3.8 and 88.6 ± 8.7 (*S. aureus*), 58.0 ± 3.7 and 55.8 ± 5.4 (*C. albicans*), and 30.2 ± 2.8 and 28.8 ± 3.3 (*A. brasiliensis*), respectively, as shown in Table 2. And there was no significant difference between 30s and 60s of spreading time. Similarly, the mean ± SD of those for 120s of spreading time were 72.0 ± 4.6 (*B. subtilis*), 24.6 ± 7.4 (*E. coli*), 22.4 ± 8.5 (*P. aeruginosa*), 84.0 ± 7.2 (*S. aureus*), 57.0 ± 4.2 (*C. albicans*), and 31.6 ± 3.9 (*A. brasiliensis*), respectively. In addition, recovery rates of *E. coli* and *P. aeruginosa* in 120s of spreading time had significant difference with that of 30s. As shown in Table 3, the recovery of each tested strain showed quite same behavior as both SCD agar and Tryptic soy agar. The recoveries of *E. coli* and *P. aeruginosa* were dramatically decreased due to 120s of spreading times regardless of media manufacturer.

The plastic rod used in this study was made by polystyrene and had a T-shape and round surface of contact face. Moreover, as a result that the contact surface of plastic rod used was buried into SCD agar and then cultured, *E. coli* was detected from used plastic rod regardless of spreading time, whereas *P. aeruginosa* was not detected from used plastic rod for only 120s of spreading time. In this study, tested strains of which recovery were decreased due to 120s of spreading time, were only gram-negative bacteria. The cell wall structure of gran-positive bacteria, yeasts and mold is comparatively thicker and rugged than that of gram-negative bacteria (Auer and Weibel, 2017, Free, 2013). Hence, it suggested that *P. aeruginosa* had a possibility to be perishable due to friction of spreading rod in spreading process. In the meantime, it suggested that long time spreading might occur to stick bacteria such as *E. coli* to plastic rod since 120s of spreading time made recovery of *E. coli* decreased even though *E. coli* could be detected from used plastic rod of all spreading time in this study. In our results, the spreading process for 120s gave the impact to recovery rate of gram-negative bacteria in performance test regardless of media manufacturer, whereas the spreading process within 60s had no impact to recovery rate of all assigned quality control microbes for performance test of SCD agar. Therefore, it suggested that within 60s of spreading time would be better for avoiding underestimate of number of microbes presented in spreading plate method in both performance test of solid agar media and practical inspection of product. And also, the verified spreading time is not important for only candidate media but also reference media since appropriate recovery rate obtained from appropriate reference number on non-selective SCD agar using spreading plate method. Even though we performed this study using pre-cultured strains, it suggested these impacts had a possibility to be stronger in case of using ready to use lyophilized strains since these strains contained injured cells due to freeze dry process. Furthermore, it seemed that friction sensitive bacteria such as *P. aeruginosa* had a stronger impact due to spreading time in selective media since injured cells which had damages in their cell wall were generically hard to grow on selective media.

In conclusion, we verified the impact to recovery rate due to spreading time in performance test of solid agar media using common SCD agar. Our results demonstrated that long spreading time had the impact to recovery rate to lead underestimation of certain bacteria. Therefore, our study suggests that spreading using plastic rod would be better to complete within 60s in spreading plate method. And it also should be verified the impact of spreading time using grass rod.

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REFERENCES

Auer, G. K. and Weibel, D. B. (2017) Bacterial Cell Mechanics. *Biochemistry*, **56**, 3710-3724.

Free, S. J. (2013) Fungal cell wall organization and biosynthesis. *Adv. Genet.*, **81**, 33-82.

International Organization for Standardization. (2007) ISO 7218. Microbiology of food and animal feeding stuffs - General requirements and guidance for microbiological examinations. International Standards Organization, Geneva, Switzerland.
International Organization for Standardization. (2013) ISO 4833-2. Microbiology of the food chain - Horizontal method for the enumeration of microorganisms - Part 2: Colony count at 30 ℃ by the surface plating technique. International Standards Organization, Geneva, Switzerland.
International Organization for Standardization. (2014) ISO 11133. Microbiology of food, animal feed and water - Preparation, production, storage and performance testing of culture media. International Standards Organization, Geneva, Switzerland.
Ministry of Health, Labor and Welfare. (2016) The Japanese pharmacopoeia, 17th ed. The Ministry of Health, Labor and Welfare, Japan.