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Case Study on the Environmental Monitoring for Biological Manufacturing Using Time-Lapse Shadow Image Analysis

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Abbreviations

RMMs, Rapid Microbiological Methods; TSIA, Time-lapse Shadow Image Analysis; VCC, Visual Colony Count; EM, Environmental Monitoring: TSA, Tryptic Soybean Casein Digest Agar, RODAC (Replicate Organism Detection And Counting) : PDA, Parenteral Drug Association

ABSTRACT

Bioburden control in the manufacturing facility is a serious concern regarding biologics due to the possibility of an significant economic impact due to batch failure from a bioburden incident. As a case study on effectively establishing a microbiological environmental monitoring program for cleanrooms, we focused on Time-lapse Shadow Image Analysis as a kind of Rapid Microbiological Method. In this study, the superior rapidity and accuracy were indicated for reference strains and environmental microbial on both 90 mm plate and RODAC plate at 25 to 30°C. Especially superior performance in the counting was observed for *B.subtilis*, *P.aeruginosa* and *A.brasiliensis*. The first and the median of colony detection speed for environmental microbial were 12 hours and 26 hours, respectively. The colony detection rate was 90% at 40 hours incubation. Additionally, the characterization of swarming behavior was recognized based on time-lapse image acquisition data at 30 minute intervals. This case study indicated that the application for environmental monitoring can contribute to reducing the bioburden excursion risk due to both the rapid detection of colonies and real-time detection for swarming behavior. TSIA would be more acceptable and easier option for biologics due to providing simple interpretations for the results and reducing the time consumption.

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1. Introduction

The various kinds of Rapid Microbiological Methods (RMMs) have been widely used for quality controls for water of pharmaceuticals, raw materials, in-process control, finished products, and microorganism identification [1-7]. Miller reported the economic benefits of moving away from the traditional method to RMMs [8]. However, RMMs cannot be straightforwardly applied for the QC (Quality Control) tests as standard methods because RMMs have different targets to measure like ATP, nucleic acid, fluorescence, the measurement principle and results, and some of them require complicated procedures and upfront costs etc. The traditional culture method has been used at many pharmaceutical manufacturing sites for the QC tests even now. In the United States Pharmacopoeia <1223> [9] and Parenteral Drug Association (PDA) Technical Report 33 [10], it emphasizes that colony counting instrumentation that replaces the human eye is do a different methodology and is subjected to qualification not validation. Like Growth Direct™ System (GDS) for the colony counting having digital imaging of microcolony autofluorescence, is one of the remarkable market instrument to operate with reduced staff [11]. The pathway for the implantation of alternate methods is relatively straight forward.

Also, recently it was known that one of the remarkable deficiencies in GMP inspection by FDA and MHRA is environmental monitoring (EM) [12, 13], and data integrity for microbiological testing is also focused by health authorities. Obviously, the paradigm of microbiological test implementation is shifting from the traditional visual colony count method (VCC) to RMMs to maintain and improve the level of cleanliness at low bioburden in the manufacturing facility, as well as the shift to isolators and restricted access barriers (RABS) from conventional cleanrooms. Recently, the practical applications of RMMs to the environmental monitoring for the manufacturing site of sterile products has been reported [14]. Also, both bioburden control for bulk drug substance and environmental monitoring for the manufacturing facilities have been reported for biological manufacturing [15-18]. Regarding the establishment of an EM program for cleanrooms, there are many different guidelines that deal with EM, such as the PIC/S Guide to Good Manufacturing Practice for medicinal products Annexes [19], USP chapter <1116> [20], the FDA Aseptic Processing Guide [21], and ISO 14698 standards [22]. To obtain a signal of a potential risk from unexpected microbial counts, EM programs, which are more crucial for biopharmaceuticals such as antibodies, vaccines, and gene therapies have been increased, and it is considered that microbial control will become increasingly important due to development of continuous manufacturing, etc. Using the RMM in EM is important to improve the operation rate of the manufacturing plants for multi-product production, to ensure high manufacturing capacity. RMMs are expected to contribute to the shortening of the startup period for biologics [23]. The alternative method for the EM application in the manufacturing facilities requires that the accuracy, consistency, standardization of procedure, convenience, and practical technology accumulation in addition to rapidity are standardized. Among these methods, we focused on the MicroBio µ3D AutoScanner system based on Time-lapse Shadow Image Analysis (TSIA) [24, 25], to detect the colony directly and rapidly on the agar plate in this study. The correlation of colony count between both TSIA and VCC for *E.coli* were already reported [25]. The data acquisition of TSIA is done automatically, detection of the colonies is extremely rapid using subsequent TSIA. It can also obtain high data reliability and data integrity about avoiding data loss, corruption and retrievability, and can also protect data against counterfeiting. On the contrary, visual colony count's data acquisition is by the naked eye and is limited based on the preset observation time points. Human errors such as misinterpretations and

misreportings might also occur.

2. Materials and Methods

2.1. Overall experimental design for the case study

For the evaluation on the feasibility of TSIA as an EM for cleanrooms PIC/S GMP grade C and D, reference strains and environmental microbial were selected. The overall experimental design was shown [Table 1].

2.1.1 Colony detection methods

2.1.1.1. Time-lapse Shadow Image Analysis (TSIA)

TSIA consisted of an incubator module, tray handling robot, image acquisition unit with CCD camera, a PC for test control, and MicroBio μ3D™ AutoScanner software. The sequential image data was taken and saved in digital storage in 30 minutes intervals during incubation. Colony count graph and the number of colonies were created periodically and automatically by software. The average, median of colony detection times, and colony detection ratio (%) for the incubation time were calculated when incubated for 5 days using by Microsoft Office Excel 2016. The colony detection ratio (%) was calculated by the ratio of number colony counts at each time point to number colony counts at the end of incubation for each reference strain based on below formula.

$$\text{Colony detection ratio (\%)} = \frac{\text{number colony counts of each time point}}{\text{number of steady counts at after 5 days incubation}} \text{ x } 100$$

2.1.1.2. Visual colony count method (VCC)

The visual colony counts were performed manually, by the naked-eye of two microbiologists after 5 days incubation, using 2X magnifying glass under fluorescent lamps.

2.1.2 Microbial samples for the test

2.1.2.1 Reference strains for optimization and demonstration

Reference strains used in this study were *Pseudomonas aeruginosa* ATCC9027, *Staphylococcus aureus* ATCC6538, *Bacillus subtilis* (*B.spizizeni*) ATCC6633, *Candida albicans* ATCC10231, and *Aspergillus brasiliensis* (*A.niger*) ATCC16404. The strains were purchased from MicroBioLogics® in the US as ready-to-use reference culture, Epower™, EZ-PEC™ and EZ-CFU™. The reference strains were stored at -20°C before use.

2.1.2.2 Environmental microbial for verification

The isolate from cleanrooms in the manufacturing facility was used in this study. The swarming bacteria was identified as *Paenibacillus* sp. by sequencing the 16S rRNA [6] and was a gram-positive, spore-forming bacteria.

The strains was stored at -80°C before use after isolated from the manufacturing facility. It was thawed at room temperature immediately prior to use. The inoculum was streaked over the agar surface and some individual bacterial cells were separated and well spaced from each other. The separated colony was inoculated at a central location on 90 mm agar plate to capture the growth forming during incubation, and then incubated at 25 to 30°C for 5 days. The image data of the growth were taken every 30 minutes during incubating.

2.1.3 Plates for the test

Two types of agar plates, RODAC plates and Tryptic Soybean Casein Digest Agar (TSA) 90 mm plate were used in this study. An appropriate microbial suspension (CFU/mL) for each strain was diluted according to instructions for use prepared by MicroBioLogics®. The inoculated sample volume for reference strain was 0.1 mL per plate. The samples were incubated at 25 to 30°C for 5 days.

2.1.3.1 RODAC plates

RODAC plates were purchased from Beckton Dickinson as gamma-sterilized, ready-to-use media supplemented with the neutralizing agents lecithin and Tween 80. The plates were stored at 4°C before use and were returned to room temperature for use.

2.1.3.2 90 mm plates

Tryptic Soybean Casein Digest Agar (TSA) were purchased from Merck KGaA as gamma-sterilized, ready-to-use media. The plates were stored at 4°C before use and were returned to room temperature for use.

2.2. Experiment for the method optimization for TSIA

The optimal colony detection parameters were discovered to establish rapidly, accurately, robustly and automatically both colony count for invisible colonies in microscopic size and the accurate colony detection while incubating the plate in 30 minutes intervals and considering the main colony detection parameters especially related to noise reduction and the threshold for the periodical colony counting. Using the example data obtained from reference strains, the colony numbers were compared before and after the colony detection parameters optimization for TSIA.

2.3. Experiment for method verification for TSIA compared to VCC

Both the first colony detection time and accuracy for colony counting were estimated using reference strains (*P.aeruginosa*, *S.aureus*, *B.subtilis*, *C.albicans* and *A.brasiliensis*) and 2 types of agar plates, i.e. RODAC plate and 90 mm plate. Traditional VCC was used as a standard method in this study. For practical application, consideration was also taken for the plates with usual low colony numbers per plate as well as unusally high colony numbers per plate as possible unexpected contamination in the manufacturing facility in EM. The suspension od

each reference strain was prepared to adjust the number of colony from 10 to 100 CFU per 0.1 mL according to instructions for use prepared by MicroBioLogics® and was inoculated on six RODAC plates and eighteen 90 mm plates. The control plates were also prepared.

2.4. Experiment for method demonstration for TSIA compared to VCC

Microbiological EM data for sampling methods such as air sampling, settle plate and surface sampling, and the samples were collected using 991 plates. One ninety six plates for air sampling, 297 plates for settle plates and 498 plates for surface sampling were used for PIC/S GMP grade C and D in the conventional cleanrooms for injectable clinical trial materials during the break of the manufacturing facility. The 90 mm plates were used for air sampling and settle plates. The passive air monitoring using Merck's MAS-100 Air Samplers was used for air sampling. The air sampling on 90 mm plate were performed at 100 L/min of flow rate for 10 minutes, in total 1000 L. The exposure time was 2 hours on 90 mm plate for settle plates. The RODAC plates were used for surface sampling. After sampling, the plates were incubated for 5 days, both methods TSIA and VCC were used for counting. Traditional VCC was used as a standard method in this study.

3. Results

In this study, compared to VCC, the method feasibility of TSIA was evaluated for the EM application, regarding method optimization, method verification and method demonstration.

3.1. Method optimization for environmental monitoring (EM)

The optimal colony detection parameters were found to establish the accurate colony detection because TSIA showed the several different colony numbers due to the difficulty in counting at around the edge of the plate. The colony detection parameters especially related to noise reductions and the thresholds for TSIA were optimized to reduce the false counts for the colonies around the edge of plate. By optimization of colony detection parameters for TSIA before EM, TSIA counted accurately and robustly the number of colonies at around the edge of the plate. The enhancement was observed by image data and colony count graph of colony counting for before and after parameter optimization [Fig.1]. In this case of *S.aureus* as an example, it was observed and the difference in counted colony number was found out before and after parameters optimization, 78 CFU and 83 CFU, respectively. The difference between default and optimal colony detection parameters was found around the edge of the 90 mm plate (shown in the white circle). In this study, the accuracy for colony counts were prioritized rather than rapidity for the colony detection when the colony detection parameters were optimized before EM. In addition, TSIA with the optimal colony detection parameters counted accurately the number of colonies at the presence of other materials such as fiber from dust-free coat, 70% ethanol for disinfectant, and cracks on the agar plate etc (data not shown).

3.2. Method verification for environmental monitoring (EM)

Focus was on both rapidity and accuracy for the method verification for reference strains. The five kinds of reference strains used for this study were counted at 30 minute intervals during incubation at 25 to 30°C with each plate as follows: *P.aeruginosa*, *S.aureus*, *B.subtilis, C.albicans* and *A.brasiliensis*. The colony numbers for each reference strain used for the test were then counted at 30 minute intervals during incubation as a total from two plates each reference strain used for the test [Table 2].

3.2.1. Estimating the first colony detection time by TSIA compared to VCC

To compare the colony detection time for environmental microbial, microbiological EM data from different sampling methods such as air sampling, contact sampling and surface sampling were collected on 991 plates in PIC/S GMP grade C and D cleanrooms. The first colony detection times were different between the microbial species and, the kind of plate used and or sampling methods [Table 2]. The first colony detection time for reference bacteria on 90 mm plate showed a faster detection time than RODAC plates. The fastest one was on both plates with *B.subtilis*. Also, the first colony detection times for bacteria samples were within 20 hours on both RODAC plate and 90 mm plate. The detection times for mold samples were significantly faster compared

to VCC. The first colony detection for *A.brasiliensi*s was 40 hours, so this was also thought speedy compared to VCC.

3.2.2. Estimating colony detection speed by TSIA and by VCC

The reference strains (*P.aeruginosa*, *S.aureus*, *B.subtilis*, *C.albicans*, and *A.brasiliensis*) on two kinds of plates were used for the estimation of colony detection speed by TSIA. The colony detection number by TSIA was estimated by the colony detection ratio (%) in the range of 10-100 CFU per plate at 25 to 30°C for each time points at 5 days incubation [Fig.2a, 2b and Table 2]. The total number of colony counts on two plates were shown at 30 minutes intervals. By TSIA, a majority of the bacteria were detected within 48 hours. It was found that the incubation condition of reference strains was at 25 to 30°C for 2 days on RODAC plate and 1 day on 90 mm plate. In the case of molds, the time point to obtain steady count would be longer than bacteria, but significantly faster than VCC, compared to traditional visual colony counts at 25 to 30°C for 5 to 7 days [26, 27].

On the other hand, *C.albicans* had slower growth, and *A.brasiliensis* showed slowest and step-wise growth. This means that large variations for colony detection time for each colony were observed. Median colony detection time of *A.brasiliensis* for both RODAC and 90 mm plate was 49.0 hours and 60.5 hours, respectively. Also, the colony detection rates were 90% at 76 hours incubation for both RODAC and 90 mm plate. In the case of molds, especially when fungi has been detected in the manufacturing site, it would be necessary to incubate the samples at 25 to 30°C for 3 days. In the case of RODAC, the detection time of RODAC was considered to be faster than that of 90 mm plate because the colony counts for *A.brasiliensis* were unaccountable due to overgrowth in such a small agar area.

3.2.3. Estimating the accuracy through colony counting results

The reference strains and environmental microbial were incubated at 25 to 30°C for 5 days on RODAC and 90 mm plate. The analysis of the data for reference strains on RODAC plates (6 plates from 1 to 200 CFU) and 90 mm plates (4 plates from 1 to 10 CFU, 10 plates from 10 to 100 CFU, 4 plates from 100-1000 CFU) obtained by both TSIA and VCC were performed. The results by TSIA and VCC for 5-days were plotted at the range of 1 to 200 CFU [Fig.3a, b]. The detail colony counts and ratio (times) of number colony counts by TSIA to number colony counts by VCC were shown [Table 3].

On RODAC plate, the counts for *P.aeruginosa* and *B.subtilis* by TSIA were 1.0 to 2.5 times and 1.0 to 1.7 times higher than those by VCC. The count for *A.brasiliensis* by TSIA was 1.6 to 2.8 times higher than those by VCC. On 90 mm plate. The counts for *B.subtilis* and *A.brasiliensis* by TSIA showed almost the same tendency with the results on RODAC plate. On the other hand, for *S.aureus* and *C.albicans* on both plates, TSIA showed almost the same results with VCC with low number to high number of colonies. In case of *S.aureus* and *C.albicans*, the results of TSIA and VCC were comparable less than approximately 300 or 400 CFU. When the number of colonies for *B.subtilis* and *C.albicans* were more than 500 CFU by TSIA, it was not counted by visual count due to too many

colonies on the agar plate [Table 3].

The data indicated that TSIA counted more accurately all samples evaluated in this study than VCC. Especially, it was shown that TSIA had far superior accuracy to VCC for microbial species which tended to expand colonies, whereas, it was impossible to count accurately the adjacent to colonies by VCC. This means that the characterization for colony formation caused a false count by VCC. Based on this data, the application of TSIA for RODAC plates would be much more effective for rapid colony detection for local contamination in the manufacturing facilities.

3.3. Method demonstration for environmental monitoring (991 plates as a total)

3.3.1. Evaluate the accuracy by TSIA for EM

Microbiological EM data for sampling methods such as air sampling, settle plates and surface sampling were collected using 991 plates as shown [Table 4]. One twelve colonies from the manufacturing facility were detected by TSIA in 19 out of 498 plates for surface sampling. The results of comparison between TSIA and VCC method in EM were shown [Fig.3c]. There was no difference between the results by both TSIA and VCC for the plate with 1 to 3 colonies in plate Nos. 1-14. On the other hand, as indicated in plate No. 18 and 19, the number of colonies by VCC were 18 CFU and 24 CFU, respectively, whereas those by TSIA were 23 CFU and 30 CFU, respectively. The data showed TSIA had far superior accuracy for counting in the case when they were adjacent to colonies on the plate.

In addition, as a representative example, the image data of microbial growth cultured on agar plate was shown in a series of images obtained at timepoints of 0, 6, 12, 18, 24, 30, 36, 48, 60, 72 and 96 hour incubation [Fig.4]. For the image data for 90 mm plate [Fig.4a], there were no adjacent to several colonies on the agar. In such a case, colony counts by both TSIA and VCC increased gradually during incubation and then, they were comparable between both TSIA and VCC as well as plate Nos. 1-14 (data not shown). The same results would be observed in many manufacturing facilities because it would be low number per plate for air sampling and settle plate. On the other hand, the image data of RODAC plates corresponding to plate No.18 in Fig.3c provided the information on colony fusions with several colonies [Fig.4b]. Also, it was observed that colony counts by TSIA increased gradually during incubation, whereas, colony counts by VCC decreased gradually during incubation (data not shown). The decrease of colony counts by VCC is due to the fusion of multiple colonies into a large colony including doublet or more colonies. Based on the image data, a false counts was indicated by VCC due to adjacency to several colonies on the surface of the plate. Additionally, false counts by VCC for plate No.18 were observed at around 24 hours incubation befor the expansion of the colonies. When tiny spots were observed, the microbiologists counted them as colonies in the worst case, whereas, the accurate counts were already impossible due to adjacency to several colonies at around over 48 hours incubation. Accurate counting colonies by VCC was impossible obviously when such a colony forming occurred for plate No.18 on RODAC plate.

3.3.2. Detect the swarming bacteria isolated from cleanrooms PIC/S GMP grade D by TSIA

Swarming behavior was observed in *Bacillus* sp., *Paenibacillus* sp. and *Pseudomonas* sp. etc. [28, 29, 30]. The swarming bacteria such as *Bacillus* sp. which was frequently observed in cleanrooms C and D. Distinguishing such kinds of microbial's behaviors rapidly and accurately is very important to control the manufacturing facilities in low bioburden, and to comprehend accurate information from the view point of GMP. In this study, in-house swarming bacteria, *Paenibacillus* sp. isolated from the manufacturing facility was used to capture the characteristic behavior in the colony growth process. By TSIA, the plate was continuously monitored and image data was acquired every 30 minutes. To acquire the image data of the growth behaviors of swarming bacteria, it was inoculated at a central location on 90 mm plate, and then cultured for 5 days at 25 to 30°C. The swarming behavior spreading to the edge of the agar plate from the central location was observed when the image data was acquired every 30 minutes by TSIA [Fig.4c]. The swarming radius exceeded 20 mm at 24 hours, and was over 60 mm at 48 hours and a majority of the surface of agar plate was covered with swarming bacteria at 96 hours. The image data acquired by TSIA contributed to distinguish swarming bacteria from unexpected contamination. On the other hand, for this *Paenibacillus* sp., it was impossible to count the number of colonies accurately by VCC at 5 days incubation, and the number of colonies was judged to be 100 CFU or more by two trained microbiologists.

4. Discussion

For the environment monitoring program, the manufacturer must capture the presence of contamination rapidly to produce the product with microbiological quality in low bioburdens. Appropriate sampling methods, frequencies, sampling timing, the numbers and volumes of sampling etc. are very important for each manufacturing facility. However, with regard to the traditional culture method by VCC, trained and qualified microbiologists are required for microbiological quality control tests. In addition, the results have been dependent on the individual's judgments and interpretation. Human errors such as misinterpretations and misreportings might also occur. Based on such a situation, recently, data integrity for microbial testing is required [31, 32]. Even if more resources were utilized, it would still be risky to assure the quality for microbial testing results completely due to insufficient data integrity.

Here, in this study, it was demonstrated that the application of automated TSIA for EM esp. cleanrooms, was able to detect and count rapidly, accurately, and reliably. TSIA could potentially solve such problems in the manufacturing facility. The points below are shown.

1. Different from the VCC, by TSIA, swarming bacteria could be specified in a series of image data acquired during incubation. For the quality assurance unit, after receiving the correct colony count number and the other information about the swarming behavior bacteria, it would be support data to make the appropriate decision regarding the impact on product quality rapidly. Especially, to prevent false judgment, it is the most superior point of this system. The colony development process could be verified easily and certainly by reviewing stored image data, going back in time, even in the middle of testing or after the test was completed.
2. Different from the VCC, by TSIA, the colony detection could be obtained within a very short time span. Especially if standardized the SOP for setting the first and final colony detection time for environmental microbial in each manufacturing facility, TSIA could contribute to an effective environment monitoring program due to the automatic colony counts.

TSIA can collect large amounts of data related to colony count, colony growth speed, detection frequency and timing and microbial behaviors per each sampling point. Originally, trained and qualified microbiologists have prior knowledge for the characterization of resident microbial in each manufacturing facility. Based on their prior knowledge, such as morphology, pigment, size, colony edge, colony shape, colony elevation, opacity and shine for colony, microbial species can be predicted without genetic identification. RMMs are very important tools when it is considering how to effectively produce many different products with limited resources, because traditional VCC of microbial counting is extremely time consuming. For the future, it would be expected to comprehend the community of environmental microbial in the manufacturing facility automatically with artificial intelligence and without excessive time-consumption due to limited resources and also from a data integrity standpoint.

5. Conclusion

As a case study on establishing effectively a microbiological environmental monitoring program for cleanrooms, using Time-lapse Shadow Image Analysis (TSIA), both rapidity and accuracy were evaluated for reference strains and environmental microbial on both 90 mm plate and RODAC plate at 25 to 30°C. Especially superior performance in the counting by TSIA was observed for *B.subtilis*, *P.aeruginosa* and *A.brasiliensis*. In this study, it was indicated that TSIA was capable of obtaining simple, rapid, accurate, and reliable data. Also, TSIA recognized the characterization of swarming behavior based on time-lapse image acquisition data. Compared to VCC, the results for RODAC plate by TSIA were far superior, and contaminated points in cleanrooms could be investigated more rapidly by TSIA. TSIA would be more acceptable for QC testing due to providing simple interpretations for the results. Microbial quality attributes would be increasingly required for biologics as well as chemical compounds. Rapid sterility testing has already been used for sterile products and cell therapies [33]. For the next new modalities such as gene therapies, the application of TSIA for environmental monitoring would contribute to improve the operation rate of the manufacturing plants for multi-product production, to ensure high manufacturing capacity due to the shortening of the startup period for biologics.TSIA would be an acceptable and easier option for biologics to reduce the time consumption, cost and resources for testing than other conventional products.

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Tables Legends

Table 1

Overall experimental design for the application of TSIA for environmental monitoring.

Type of sampling	Type of plate	Optimization	Verification	Demonstration
Air sampling	90 mm plate	RS[1]	RS[1]	EM[2]
Settle plate	90 mm plate			EM[2]
Surface sampling	RODAC plate	RS[1]	RS[1]	EM[2]

[1] Reference strains, [2] Environmental microbial

Table 2

Colony detection time by TSIA for reference strains and environmental microbial

Microorganism		Total colony numbers (CFU) / plates numbers	Type of plate	First detection time (hours)	Detection time (hours) at median of detection ratio	Detection time (hours) at 90% of detection ratio	Average detection time (hours)
Reference strain							
(Bacteria)	*P.aeruginosa*	44 / 2	RODAC	20.0	24.0	51.0	30.3 ± 14.8
		57 / 2	90 mm	18.0	20.0	24.0	20.7 ± 2.6
	S.aureus	44 / 2	RODAC	20.0	23.0	30.0	25.1 ± 5.3
		112 / 2	90 mm	15.5	21.0	26.0	22.9 ± 5.8
	B.subtilis	50 / 2	RODAC	15.0	19.0	34.0	23.8 ± 13.0
		158 / 2	90 mm	13.5	17.0	22.0	17.9 ± 3.8
(Mold)	*C.albicans*	40 / 2	RODAC	25.5	30.5	47.5	36.0 ± 11.4
		68 / 2	90 mm	22.0	27.5	37.0	29.0 ± 5.2
	A.brasiliensis	54 / 2	RODAC	35.5	49.0	76.0	53.6 ± 15.0
		39 / 2	90 mm	40.0	60.5	76.0	60.6 ± 12.6
Environmental microbial (Bacteria)		112 /19	RODAC	12.0	26.0	40.0	30.3 ± 13.1
		9 / 4	90 mm[1]	19.0	22.0	NA	25.9 ± 7.9
		4 / 3	90 mm[2]	20.0	28.5	NA	31.5 ± 12.7

[1]Air sampling, [2]Settle plates

Table 3

Comparison of colony counting by TSIA and VCC for reference strains

Reference strains	Colony counts (CFU) by TSIA / by VCC on RODAC plate	Total plate numbers	Ratio (times)	Colony counts (CFU) by TSIA / by VCC on 90 mm plate	Total plate numbers	Ratio (times)
P.aeruginosa	5-90 / 2-68	6	1.0-2.5	3-341 / 3-268	18	1.0-1.4
S.aureus	3-144 / 3-143	6	1.0	1-302 / 1-313	18	1.0-1.2
B.subtilis	4-234 / 4-134	6	1.0-1.7	6-153 / 6-93	14	1.0-1.7
				492-562 / NA	4	-
C.albicans	3-172 / 3-171	6	1.0	5-383 / 5-375	16	1.0-1.2
				592-659 / NA	2	-
A.brasiliensis	11-163 / 5-109	6	1.6-2.8	2-121 / 2-98	18	1.0-2.5

NA: not available (uncountable)

Table 4

Method demonstration for environmental monitoring (991 Plates as a Total)

Type of sampling	Type of plate	Total plate numbers	Detected plate numbers	Colony counts (CFU) by TSIA / by VCC
Air sampling	90 mm	196	4	9 / 9
Settle plate	90 mm	297	3	4 / 4
Surface sampling	RODAC	498	19	112 / 99

Figure legends

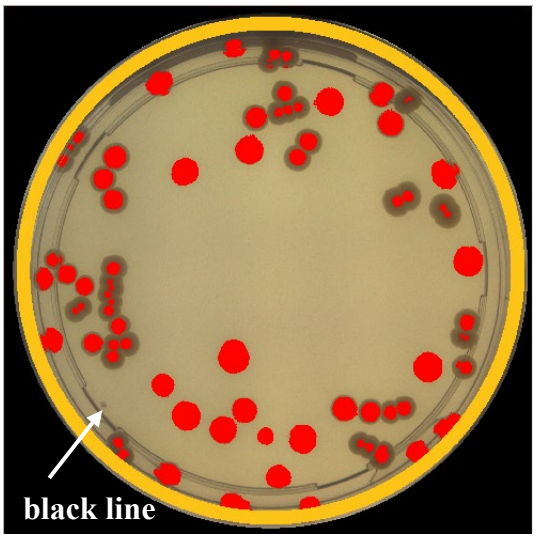
Fig.1 Colony detection parameters optimization for TSIA. Image data and colony count graph of (a) before and (b) after colony detection parameters optimizations for e.g. *S.aureus* incubated at 25 to 30°C for 5 days were shown. Red spots indicated the recognized colonies by MicroBio μ3D™ AutoScanner software, and the outsides of black lines are the edge area of plate. White circles pointed out the edge area makes the differences in colony detections for before and after.

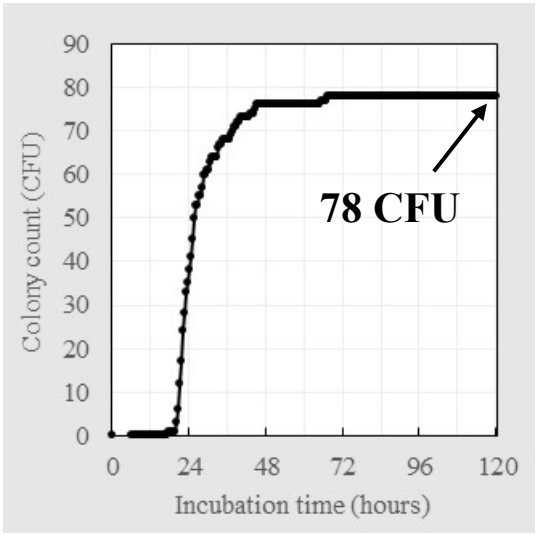
Fig.2 Colony detection speed by TSIA for reference strains and environmental microbial. The reference strains and environmental microbial were incubated at 25 to 30°C for 5 days. (a) Colony detection graphs for reference strains and environmental microbial on RODAC plate were shown. (b) Colony detection graphs for reference strains on 90 mm plate were shown; *P.aeruginosa* (pink lines), *S.aureus* (blue lines), *B.subtilis* (red lines), *C.albicans* (brown lines), *A.brasiliensis* (green lines), Environmental microbial (orange lines).

Fig.3 Comparison of colony counting by TSIA and VCC for reference strains and environmental microbial. The reference strains and environmental microbial were incubated at 25 to 30°C for 5 days on (a) RODAC and (b) 90 mm plate. The number of colony counts obtained by TSIA and VCC on each plate in parallel were plotted in the range from 1 to 200 CFU. The results by TSIA and VCC (n=6 for RODAC, n=14-18 for 90 mm) were plotted by closed symbols and open symbols, respectively: Bacteria (left panels); *P.aeruginosa* (pink square), *S.aureus* (blue triangle), *B.subtilis* (red circle), Molds (right panels); *C.albicans* (brown rhombus), *A.brasiliensis* (green circle). (c) The number of colony counts obtained by TSIA and VCC from environmental monitoring were shown by closed bars and open bars (N=19)

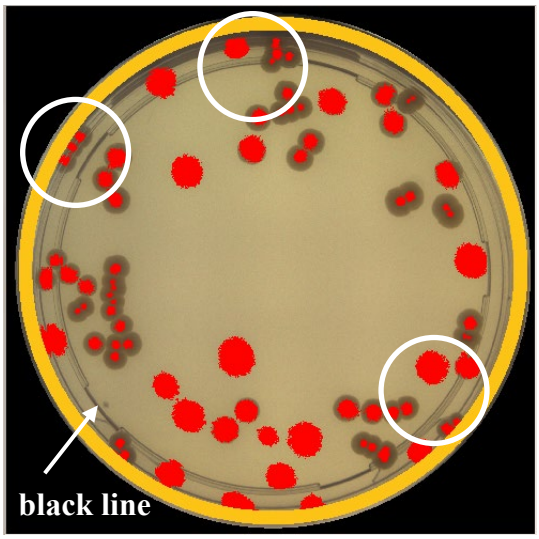
Fig.4 Representative sequential image data of environmental microbial by TSIA. (a) Image data of environmental microbial on 90 mm plate were shown. (b) Image data of environmental microbial on RODAC plate from plate No.18 of Fig.3c were shown. White circles pointed out the decrease of colony counts by VCC due to the fusion of multiple colonies including doublet or more colonies during incubation. (c) Image data of swarming bacteria on 90 mm plate were shown. The swarming radius exceeded 20 mm at 24 hours, and was over 60 mm at 48 hours, and a majority of the surface of agar plate was covered with swarming bacteria at 96 hours.

(a)


black line


Colony count (CFU)
90
80
70
60
50
40
30
20
10
0
0
24
48
72
96
120
Incubation time (hours)
78 CFU

(b)


black line

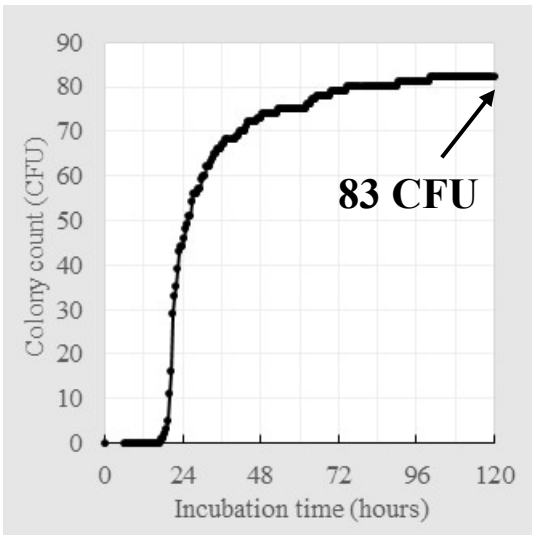
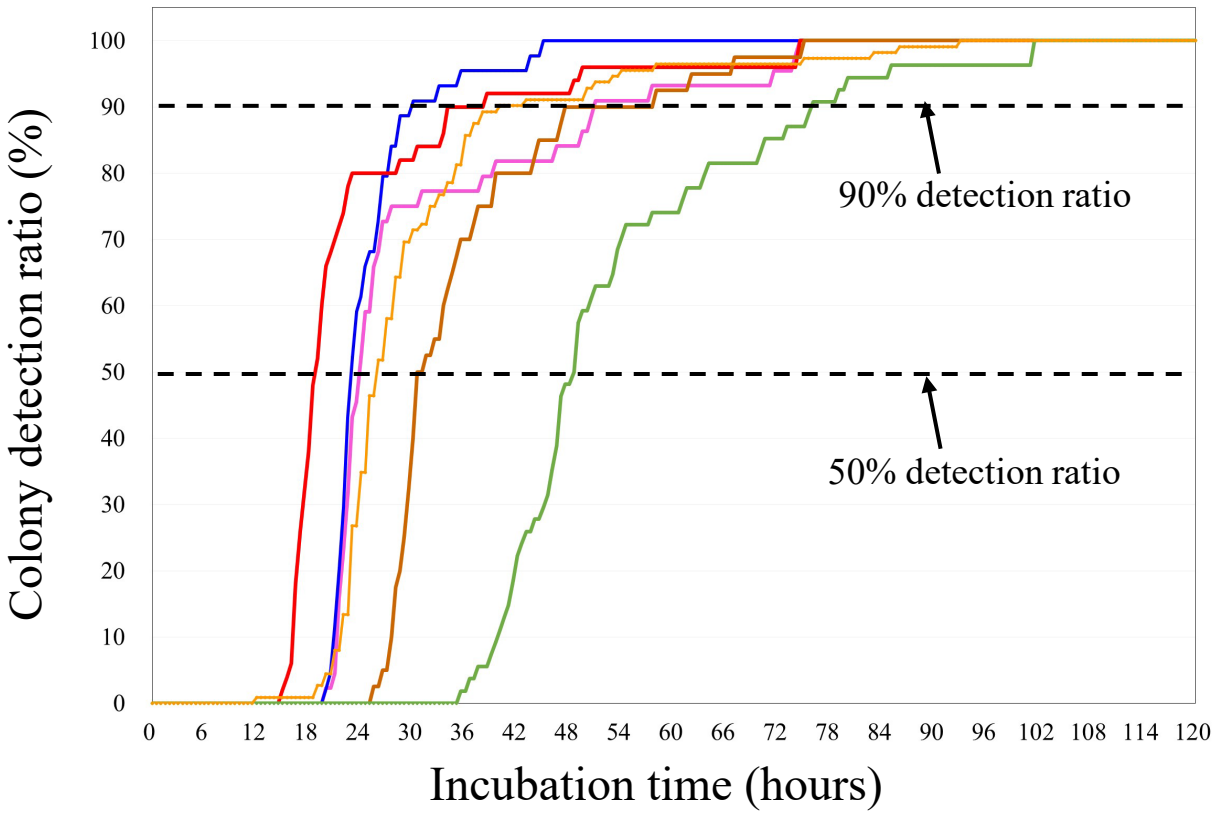

Colony count (CFU)
90
80
70
60
50
40
30
20
10
0
0
24
48
72
96
120
Incubation time (hours)
83 CFU

Fig.1

(a)


Colony detection ratio (%)
100
90
80
70
60
50
40
30
20
10
0
90% detection ratio
50% detection ratio
0 6 12 18 24 30 36 42 48 54 60 66 72 78 84 90 96 102 108 114 120
Incubation time (hours)

(b)

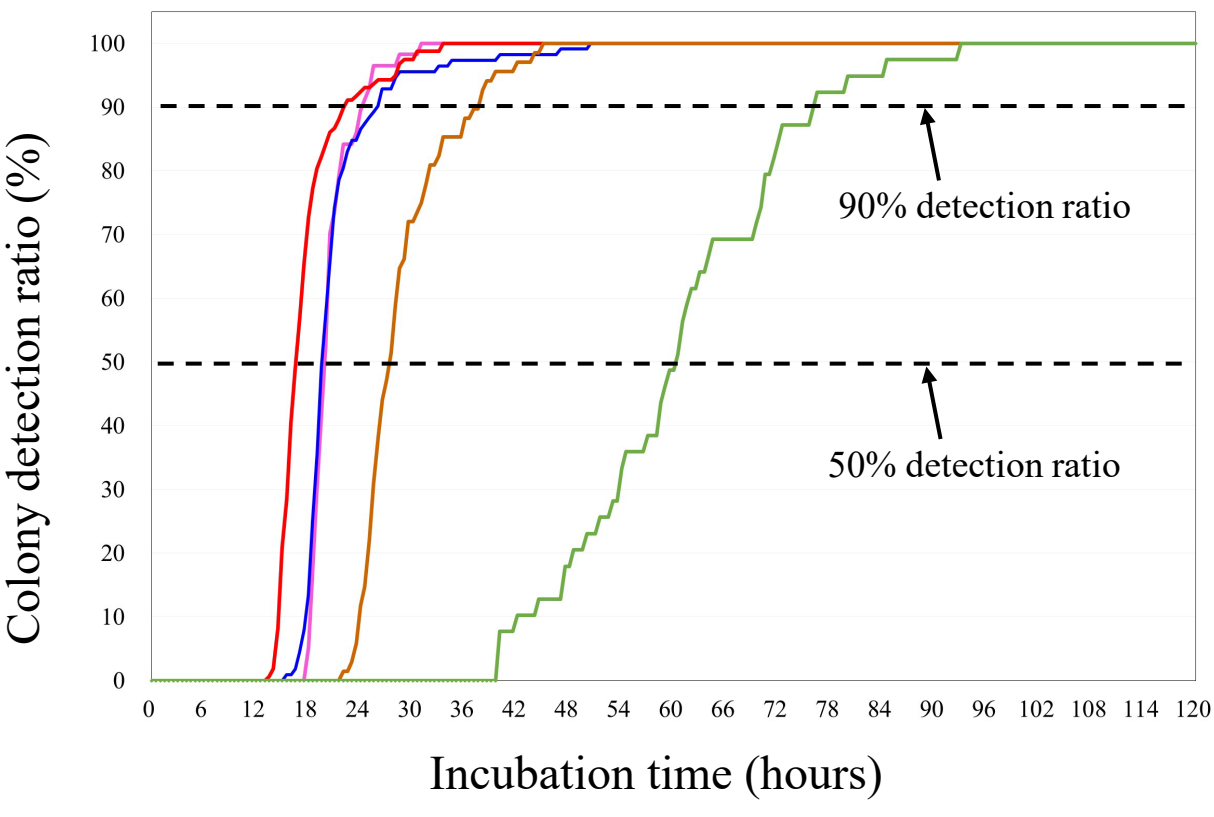

Colony detection ratio (%)
100
90
80
70
60
50
40
30
20
10
0
90% detection ratio
50% detection ratio
0 6 12 18 24 30 36 42 48 54 60 66 72 78 84 90 96 102 108 114 120
Incubation time (hours)

Fig.2

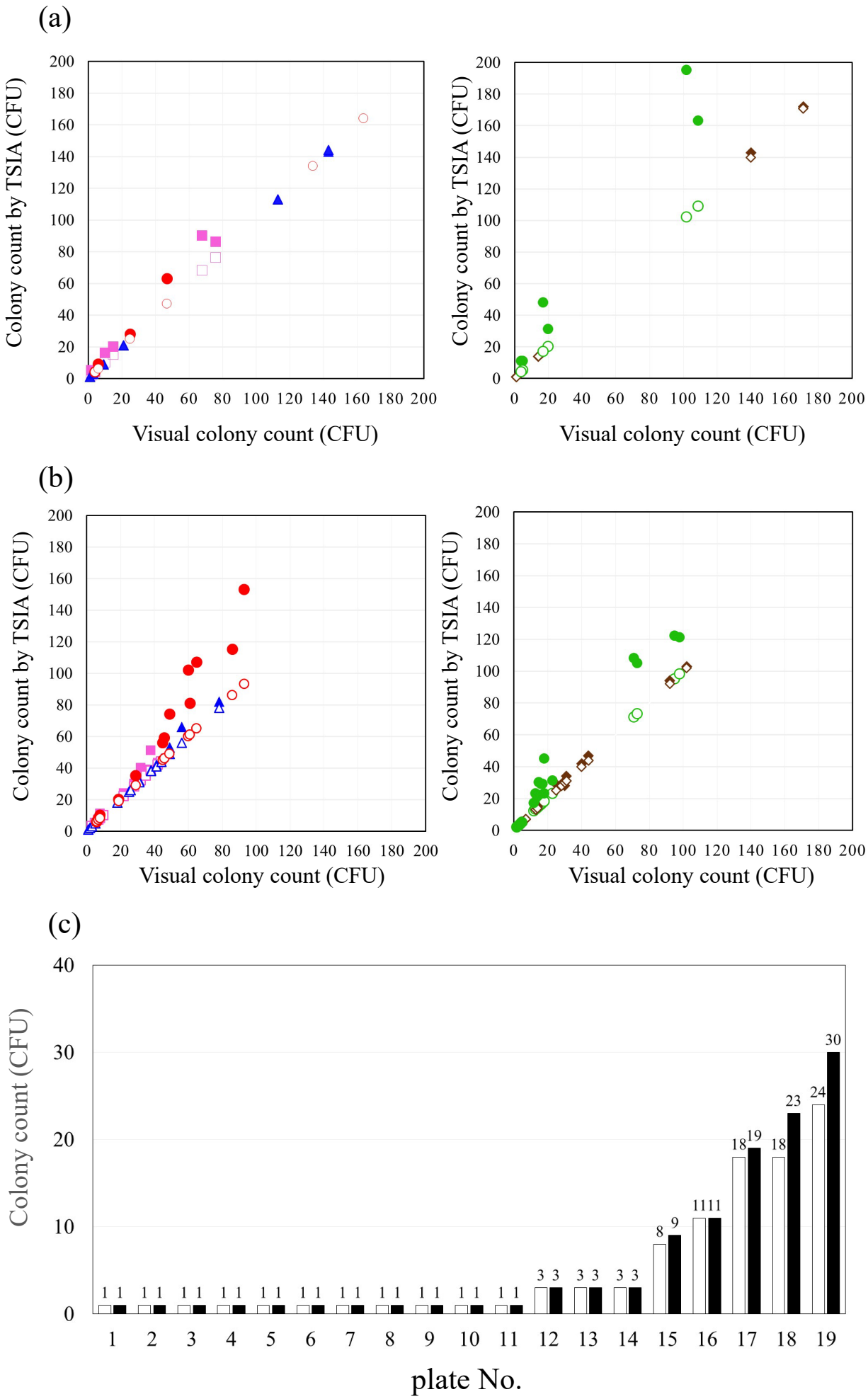

(a)
Colony count by TSIA (CFU)
Visual colony count (CFU)
(b)
Colony count by TSIA (CFU)
Visual colony count (CFU)
(c)
Colony count (CFU)
plate No.

Fig.3

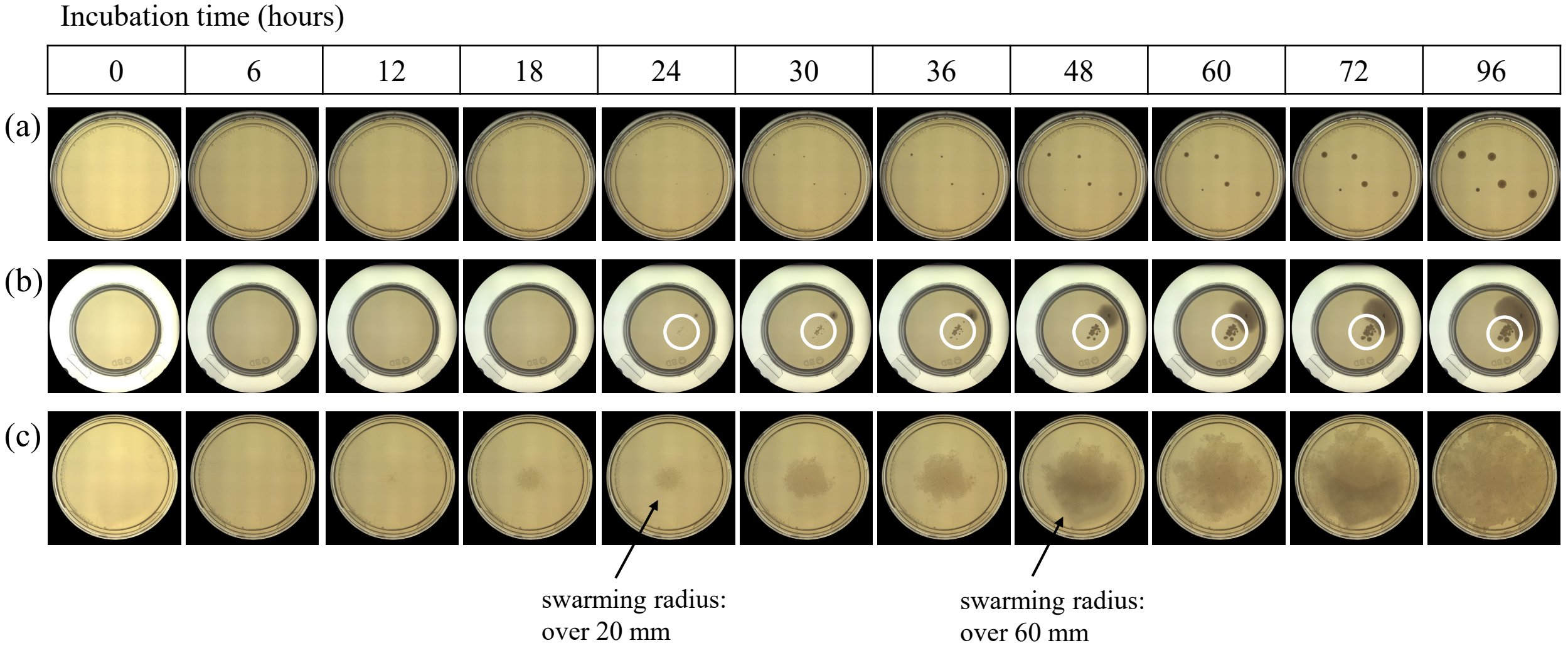

Incubation time (hours)
0
6
12
18
24
30
36
48
60
72
96
(a)
(b)
(c)
swarming radius:
over 20 mm
swarming radius:
over 60 mm

Fig.4