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Verification of a simplified disk diffusion method for antimicrobial susceptibility testing of bovine mastitis isolates

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Abstract

Bovine mastitis is mainly treated with antimicrobials. Determination of antimicrobial treatments based on the results of an antimicrobial susceptibility test is important to reduce the risk of emergence of antimicrobial resistance and to provide effective treatment. In Japan, not only the standardized agar disk diffusion method (standardized-ADD) based on Clinical and Laboratory Standards Institute guidelines, but also the agar disk diffusion method further simplified (simplified-ADD) are widely used as antimicrobial susceptibility tests for bovine mastitis isolates in the clinical laboratory. However, whether the simplified-ADD is a useful alternative to the standardized-ADD has not yet been sufficiently verified. Therefore, to verify the usefulness of the simplified-ADD, we compared the results of the standardized-ADD and the simplified-ADD using clinical isolates of bovine mastitis. Following testing of 83 isolates from 11 bacterial species, the correlation coefficient of the disk zone diameters in both methods was 0.92, indicating that the simplified-ADD is effective as an alternative method to the standardized-ADD. However, because the disk zone diameter tended to be smaller in the simplified-ADD than in the standardized-ADD, sufficient attention should be paid to this point when determining the treatment for clinical cases of mastitis from the results of the simplified-ADD. The fact that a difference in the results between the two methods was present means that the results cannot be interpreted based on the same criteria. Therefore, determination of the criteria appropriate for the simplified-ADD is needed.

Key Words: agar disc diffusion method, antimicrobial susceptibility test, bovine mastitis, Clinical and Laboratory Standards Institute

Introduction

Bovine mastitis is one of the most frequent diseases in dairy cows, and is a costly disease that results in large economic loss due to decreased lactation, milk disposal, and increased treatment costs[13,24]. Bovine mastitis is inflammation of the udder due to microbial infection and is mainly treated with antimicrobials[5,12].

The use of antimicrobials in livestock directly and indirectly induces a selective pressure for antimicrobial resistance in certain conditions, and the amount of drug used and resistance are positively correlated[10]. Therefore, when determining the antimicrobial treatment for clinical cases, selection of effective antimicrobials

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due to accurate identification of the causative microorganism and an accurate antimicrobial susceptibility test is important to reduce the risk of resistance due to inappropriate use of antimicrobials[15,17]. Although no direct scientific evidence supports the claim that the use of antimicrobials for the treatment of bovine mastitis increases the emergence and prevalence of resistance[19], some studies suggest that drug use in adult dairy cows may provide selective pressure for the emergence of resistance[22,27]. On the other hand, in a survey that monitored the antimicrobial susceptibility of bovine mastitis isolates over time in various regions, no significant change in susceptibility was reported[4,19]. However, this fact does not exclude the possibility that the use of antimicrobials for the treatment of bovine mastitis induces the emergence and prevalence of resistance. Prudent use of antimicrobials in dairy farms is a very important countermeasure to development of worldwide resistance in livestock and in the veterinary and medical fields[19].

Identification of resistant strains is important for effective treatment selection. The cure rate of mastitis caused by resistant strains is significantly lower than that of susceptible strains for some pathogenic species[25,26]. Herd-level knowledge of recent susceptibility patterns may also be useful to guide treatment decisions for some pathogens[21].

Several methods are used for antimicrobial susceptibility testing such as disk-diffusion, well diffusion, and broth or agar dilution tests[3]. The agar disk diffusion method based on Clinical and Laboratory Standards Institute (CLSI) guidelines[7,8], which are internationally standardized methods, is a widely adopted antimicrobial susceptibility test for bovine mastitis isolates in the clinical laboratory. Several issues have been indicated regarding the interpretation of in vitro antimicrobial susceptibility for determining treatments of clinical cases. The issues are that most antimicrobials used for treatment of bovine mastitis are selected with criteria based on medical knowledge, and the relationship among the susceptibility pattern of bovine mastitis isolates, the behavior of antimicrobials in mastitis milk, and clinical outcomes has not been fully considered[2,9,18,20]. One study found no correlation between antimicrobial susceptibility and the cure rate of bovine mastitis[11], possibly because bovine mastitis is caused by multiple factors, and its prognosis depends on many factors. These issues sometimes induce the discussion about the need for antimicrobial susceptibility testing in bovine mastitis. In addition, antimicrobial susceptibility tests are costly and not recommended from a cost-benefit standpoint[6,23]. Assuming that it is carried out at one clinic level, the agar disk diffusion method procedure is complicated and requires many materials, thus limiting its implementation. In fact, a report has shown that the antimicrobial susceptibility test implementation rate for food-producing animals in Europe is less than 50%[6].

However, as mentioned above, proper use of antibacterial agents is required due to the issue of antimicrobial resistance. In addition, antimicrobial susceptibility tests can provide important basic data for guiding the treatment of clinical cases due to comprehensive interpretation in combination with other data such as rearing management, milking hygiene, treatment history, or clinical symptoms. Considering the above, antimicrobial susceptibility testing in bovine mastitis is important and useful, but the complexity of the procedure remains a problem.

The organization of the livestock insurance system in Japan recommends not only the standardized agar disk diffusion method (standardized-ADD) based on CLSI guidelines, but also agar disk diffusion further simplified (simplified-ADD) as methods for antimicrobial susceptibility tests for bovine mastitis isolates in the clinical laboratory[16]. The simplified-ADD prioritizes reduction of examination time and cost by simplifying the procedure of preparing the inoculum and unifying culture conditions for each bacterial species. Currently, in Japanese cattle

clinical practice, few regions have examination centers or other systems for intensive and rapid bacteriological examinations, and in most regions, veterinarians have no choice but to perform clinical examinations at the level of one clinic. In clinics that mainly perform medical services, the personnel, time, equipment, and costs that can be devoted to clinical examinations are limited, and more economical and simpler procedures are required. The simplified-ADD is widely carried out in clinical practice in Japan as a feasible method under such circumstances.

However, whether the simplified-ADD is useful as an alternative to the standardized-ADD has not yet been sufficiently verified. Therefore, to verify the usefulness of the simplified method, we compared the results of the standardized-ADD and the simplified-ADD using clinical isolates of bovine mastitis.

Materials and Methods

Collection of milk samples and identification of mastitis pathogens

A total of 83 strains isolated from the milk of bovine clinical mastitis cases that occurred in Japan from 2013 to 2016 were tested, including the following: 10 *Staphylococcus aureus*, 5 *Staphylococcus saprophyticus*, 5 *Staphylococcus xylosus*, 10 *Streptococcus dysgalactiae*, 10 *Streptococcus uberis*, 6 *Enterococcus faecalis*, 6 *Enterococcus faecium*, 11 *Trueperella pyogenes*, 10 *Escherichia coli*, 2 *Klebsiella oxytoca*, and 8 *Klebsiella pneumoniae*. Milk samples (10 µL) were individually added to 5% sheep blood agar and cultured aerobically at 37°C for 24 hr, according to the method of the National Mastitis Council[1]. The obtained strains were molecularly identified with PCR and sequencing. Bacterial genomic DNA was isolated with InstaGene Matrix (Bio-Rad Laboratories Inc., Hercules, CA, USA). To amplify 16S rRNA, PCR was performed with the following primers: 16S F1 sense primer, 5'-TTCCCGGGTCTTGTACACAC-3'; 23S R1 antisense primer, 5'-TTGTAACTCCGTATAGAGTGTCC-3' (for Staphylococci); 27F sense primer, 5'-AGAGTTTGATCCTGGCTCAG-3'; 1492R antisense primer, 5'-GGTTACCTTGTTACGACTT-3' (for bacteria except Staphylococci). PCR conditions were as follows: denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 1 min for 35 cycles. Amplified DNA was isolated from agarose gels using Quantum Prep Freeze'N Sequeeze DNA Gel Extraction Spin Columns (Bio-Rad Laboratories Inc.) and purified with QIAquick PCR purification kit (Qiagen). Purified products were sequenced directly using the Big Dye Terminator v3.1 (Applied Biosystems, Waltham, MA, USA) on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems).

Agar disk diffusion test

Antimicrobial agents: Both methods were performed with antimicrobial disks of benzylpenicillin (PCG), oxytetracycline (OTC), kanamycin (KM), cefazolin (CEZ) (Nippon Becton Dickinson Co., Ltd., Tokyo, Japan), pirlimycin (PLM), enrofloxacin (ERFX) (Eiken Chemical Co., Ltd., Tokyo, Japan), and marbofloxacin (MBFX) (Vetoquinol, France).

Gram-positive bacteria were tested with PCG, OTC, KM, CEZ, PLM, ERFX, and MBFX, and Gram-negative bacteria with OTC, KM, CEZ, ERFX, and MBFX.

Standardized-ADD: Standardized-ADD was carried out based on the CLSI guidelines[7,8] and the protocol of the commercial disks using 83 strains. In brief, each inoculum was prepared by dissolving colonies in sterile saline and adjusting the suspension to achieve a turbidity equivalent to a 0.5 McFarland standard. In this study, we used a calibrated photometric device (Waken B Tech Co., Ltd., Kyoto, Japan) to adjust the turbidity. The adjusted suspension was used to inoculate a testing plate with a cotton swab, and

Table 1. The test conditions of the standardized-ADD and the simplified-ADD.

a. Standardised-ADD

Strains	Culture media	Cultural conditions	
Enterococcus spp.	MHA	PCG, OTC, KM, CEZ: 24hr ERFX, PLM: 16-18hr	35°C
Streptococcus spp.	MHA with 5% Sheep Blood	20-24hr	35°C, 5%CO_2
Trueperella pyogenes	MHA with 5% Sheep Blood	PCG, OTC, KM, CEZ: 16-24hr ERFX, PLM: 24hr	35°C
Stapylococcus spp.	MHA	PCG, OTC, KM, CEZ: 24hr ERFX, PLM: 16-18hr	35°C
Gram-negative bacteria	MHA	PCG, OTC, KM, CEZ: 16-24hr ERFX, PLM: 16-18hr	35°C

b. Simplified-ADD

Strains	Culture media	Cultural conditions		Number of colonies
Enterococcus spp.	5% Sheep Blood Agar	24hr	37°C	20~30
Streptococcus spp.	5% Sheep Blood Agar	24hr	37°C	20~30
Trueperella pyogenes	5% Sheep Blood Agar	24hr	37°C	50~100
Stapylococcus spp.	MHA	24hr	37°C	2~3
Gram-negative bacteria	MHA	24hr	37°C	1/8~1/4

MHA: Mueller Hinton Agar.
PCG: Benzylpenicillin; OTC: Oxytetracycline; KM: Kanamycin; CEZ: Cefazolin; PLM: Pirlimycin; ERFX: enrofloxacin; MBFX: marbofloxacin.

the inoculum was spread at different angles three times in 15 min. Antimicrobial disks were placed onto the test plates 3-5 min after inoculation but no more than 15 min, and incubation of the testing plates began within 15 min after placing the disks. Quality control tests were performed using *E. coli* ATCC25922. After culturing in appropriate conditions for each bacterial species, the zone disk diameter was measured with a caliper. The medium and the culture conditions are described in Table 1.

Simplified-ADD: Simplified-ADD was performed based on the procedure stipulated in the Japanese livestock insurance system[16] with some modification using 83 strains in this study. For this method, instead of preparing each inoculum, the number of colonies determined for each bacterial species was directly picked up with a sterile cotton swab and spread directly on a testing plate at different angles three times. In particular, 2-3 colonies for *Staphylococcus* spp., 20-30 for *Streptococcus* spp. and *Enterococcus* spp., 50-100 for *Trueperella pyogenes*, and 1/8-1/4 for Gram negative bacteria were picked up for inoculation. Testing plates were made with Mueller Hinton Agar for *Staphylococcus* spp. and Gram-negative bacteria, and 5% Sheep Blood Agar for *Enterococcus* spp., *Streptococcus* spp., and *Trueperella pyogenes*. Antimicrobial disks were placed within minutes, and the culture was started within 15 min. The incubation conditions were unified for all bacterial species in this method, with aerobic culturing at 37°C for 24 hr. After culturing, the disk zone diameter was measured with a caliper. The number of colonies determined for each bacterial species, the medium, and the culture conditions are described in Table 1. Two modifications were made in this study that differed from the procedure stipulated in the Japanese livestock insurance system. First, because the incubation time was not specified, we selected 24 hr. Second, we changed the test medium for *Enterococcus* spp. from Mueller Hinton Agar to 5% Sheep Blood Agar because the colony morphology of Enterococcus spp. is similar to that of Streptococcus spp. and is difficult to distinguish on one clinic level.

Table 2. The alternative ranges of quality control in the simplified-ADD.
Each range was calculated from the mean zone diameter ± 2×SD.

Strains	The alternative ranges of quality control (mm)						
	PCG	OTC	KM	CEZ	PLM	ERFX	MBFX
Escherichia coli ATCC25922	-	18.0 - 21.7	17.2 - 21.1	17.6 - 21.4	-	26.4 - 31.2	29.6 - 32.9
Staphylococcus aureus ATCC25923	8.6 - 12.6	19.7 - 22.6	16.0 - 20.9	22.4 - 26.4	12.9 - 16.7	21.5 - 23.7	20.0 - 23.6

- : Not tested.
PCG: Benzylpenicillin; OTC: Oxytetracycline; KM: Kanamycin; CEZ: Cefazolin; PLM: Pirlimycin; ERFX: enrofloxacin; MBFX: marbofloxacin.

At present, no method has been established for guaranteeing quality control in the simplified-ADD. In this study, to confirm the reproducibility of the simplified-ADD, we performed a preliminary test using eight strains (*E. coli* ATCC25922, *Staphylococcus aureus* ATCC25923, and six isolates from bovine clinical mastitis cases including *Staphylococcus aureus*, *Streptococcus uberis*, *Enterococcus faecalis*, *Trueperella pyogenes*, *Escherichia coli*, and *Klebsiella pneumoniae*). Preliminary tests were performed with five replicates using five individual inoculum preparations for two consecutive test days based on the simplified-ADD. Confirming the reproducibility from the results of each strain, the mean zone diameter ± 2×SD of the ATCC strains was used as an alternative to the quality control range for the simplified-ADD in this study.

Statistical analysis

The statistical analysis was conducted using the intraclass correlation coefficient to confirm the reproducibility of the simplified-ADD, and Spearman's rank correlation coefficient was used for comparison of the two methods.

Results

As a result of the preliminary test with eight strains, the intraclass correlation coefficient was 0.97, confirming the reproducibility. The alternative ranges of quality control in the simplified-ADD are described in Table 2.

The results of the disk diffusion method for a total of 83 isolates using the standardized-ADD and the simplified-ADD are shown in Figure 1 and Table 3. The correlation coefficient of the disk zone diameter in the standardized-ADD and the simplified-ADD of 83 isolates was 0.92 ($P < 0.001$). The disk zone diameter tended to be smaller in the simplified-ADD than in the standardized-ADD.

Discussion

The simplified-ADD has become widespread as an antimicrobial susceptibility test for bovine mastitis in Japanese clinics. However, evidence for the validity of the protocol has not been published. Therefore, the results of the standardized-ADD and the simplified-ADD were compared to verify the usefulness of the simplified-ADD in this study. The correlation coefficient of the disk zone diameter was as high as 0.92, demonstrating that the simplified-ADD is effective as an alternative method to the standardized-ADD.

On the other hand, the disk zone diameter tended to be smaller in the simplified-ADD than in the standardized-ADD. The most influential factor is not clear, but the inoculum size, type of medium, and culture conditions were different between the two methods, and these differences may cause the tendency for a smaller disk zone diameter in the simplified-ADD.

Because the disk zone diameter tended to be smaller in the simplified-ADD, sufficient

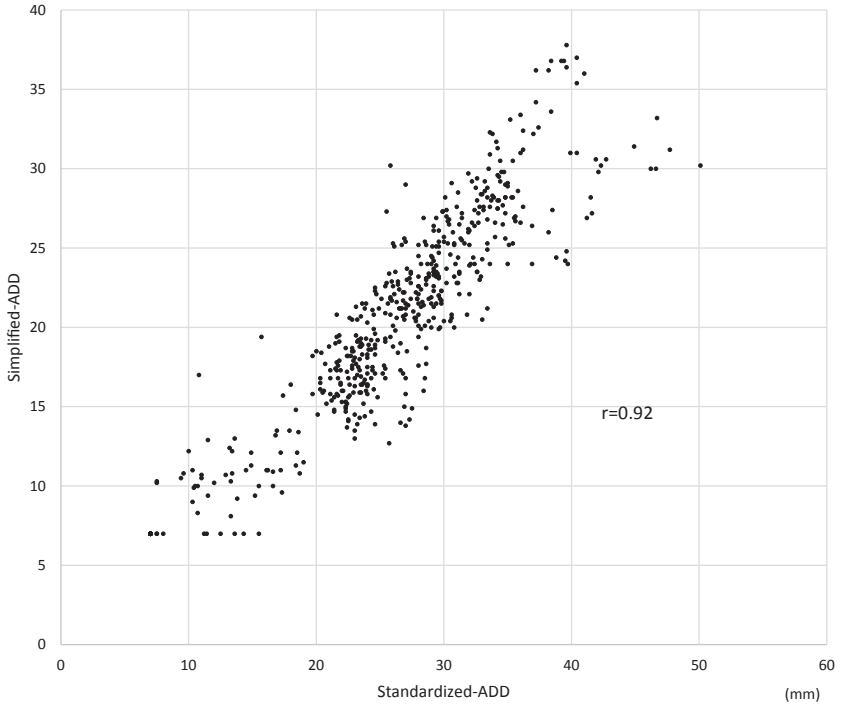

Fig. 1.
The disk zone diameter of the standardized-ADD and the simplified-ADD of 83 isolates. The correlation coefficient was 0.92 ($P < 0.001$).

careful attention should be paid to this point when determining the treatment for clinical cases of mastitis from the results of the simplified-ADD. In other words, the fact that a difference in the results was present between the two methods means that the results cannot be interpreted based on the same criteria. If the result of the simplified-ADD is judged by the criteria of the standardized-ADD, an erroneous result may be obtained. Although establishing appropriate criteria for each procedure of antimicrobial susceptibility testing is necessary, at present, no criteria have been determined for applying the simplified-ADD for the selection of antimicrobials for clinical cases of bovine mastitis. Ideally, establishing criteria for a high therapeutic effect is desirable by monitoring the antimicrobial susceptibility of clinical isolates of bovine mastitis in each region over time and accumulating knowledge of pharmacokinetics and pharmacodynamics in mastitis cows, clinical breakpoints in field trials, and conditions that induce resistance in the treatment of bovine mastitis. In addition, as each method leads to different results, the criteria should be considered in detail for each method.

Just as important as the determination of criteria is determination of how to guarantee quality control in the simplified-ADD. Ideally, setting the quality control range using standard strains is desirable. However, because the simplified-ADD is supposed to be performed at the level of one clinic, considering a simple procedure for quality control that can be realized in the field even if it is not the ideal procedure for quality control is necessary. At a minimum, proper storage of the medium and discs and strict adherence to the protocol when carrying out the simplified-ADD are necessary. When the quality control range is set, performing occasional outsourced inspections and comparing the results with the results in their own laboratory is also

Table 3. The disc zone diameter in the standardized-ADD and the simplified-ADD for each antimicrobial and strain.

Strains (Number of isolates)	Mean disc zone diameter ± SD (mm)													
	PCG		OTC		KM		CEZ		PLM		ERFX		MBFX	
	Stand	Simp	Stand	Simp	Stand	Simp	Stand	Simp	Stand	Simp	Stand	Simp	Stand	Simp
S. aureus (10)	39.3±1.1	36.5±0.6	28.3±0.9	21.8±1.5	23.9±1.0	18.1±0.9	34.7±1.2	31.5±1.2	23.5±1.0	17.2±1.4	29.5±0.7	24.0±1.2	25.8±0.7	22.4±0.7
S. saprophyticus (5)	34.0±1.9	25.3±3.8	30.6±1.6	23.3±1.0	32.7±0.8	28.5±0.9	34.6±0.9	28.3±0.8	25.8±4.4	20.7±2.7	28.8±1.0	22.2±0.7	26.5±2.5	21.6±0.5
S. xylosus (5)	32.9±1.5	22.7±1.7	19.5±8.7	15.5±7.0	33.0±1.2	26.8±1.5	35.6±0.6	27.5±0.7	21.7±5.3	17.6±4.0	28.9±1.1	23.0±0.5	26.9±0.8	22.1±0.6
S. dysgalactiae (10)	33.8±1.8	27.4±1.2	21.6±1.0	17.3±0.8	11.2±1.9	9.5±1.4	31.7±1.3	24.4±1.7	23.7±0.7	16.8±1.2	22.9±1.0	15.5±1.4	22.5±0.9	15.6±0.9
S. uberis (10)	28.9±2.4	26.2±1.7	22.3±7.1	17.6±4.2	9.6±3.5	8.9±3.2	28.1±1.6	24.4±2.2	20.5±6.2	15.1±3.4	25.3±1.6	17.2±2.0	23.6±1.9	16.8±1.5
E. faecalis (6)	23.2±1.1	17.3±1.4	11.6±6.2	11.7±2.6	7.6±1.4	7.5±1.1	18.6±2.0	13.1±1.8	7.2±0.4	7.0±0.0	21.7±1.6	15.3±2.2	20.3±1.0	15.1±1.8
E. faecium (6)	24.9±4.5	14.6±2.3	27.5±6.4	19.2±3.2	14.8±1.8	9.0±2.4	7.2±0.2	7.0±0.0	26.5±2.1	15.6±1.4	15.9±2.7	10.2±2.1	18.9±3.2	12.5±2.4
T. pyogenes (11)	44.5±3.1	35.4±1.9	30.7±6.2	25.7±3.4	29.0±1.1	24.4±1.9	39.8±0.6	26.3±2.2	33.5±1.4	26.6±1.9	28.8±1.6	21.9±1.9	29.4±0.9	21.1±1.2
E. coli (10)	-	-	15.4±8.5	13.3±6.4	22.8±0.7	19.2±1.0	25.8±1.2	20.9±1.4	-	-	32.2±1.5	27.0±1.2	35.5±1.6	31.3±1.7
K. oxytoca (2)	-	-	25.9±0.0	21.9±1.1	22.9±0.2	19.8±1.6	22.8±1.2	20.6±0.3	-	-	30.2±0.3	26.9±0.4	32.3±0.4	28.9±0.9
K. pneumoniae (8)	-	-	19.2±7.2	17.1±6.0	19.9±4.9	16.6±3.7	21.5±8.4	18.5±6.7	-	-	28.1±0.9	23.4±1.1	30.3±0.8	26.9±0.9

Stand: standardized-ADD; Simp: simplified-ADD.
- : Not tested.
PCG: Benzylpenicillin; OTC: Oxytetracycline; KM: Kanamycin; CEZ: Cefazolin; PLM: Pirlimycin; ERFX: Enrofloxacin; MBFX: Marbofloxacin; *S. aureus*: *Staphylococcus aureus*; *S. saprophyticus*: *Staphylococcus saprophyticus*; *S. xylosus*: *Staphylococcus xylosus*; *S. dysgalactiae*: *Streptococcus dysgalactiae*; *E. faecalis*: *Enterococcus faecalis*; *E. faecium*: *Enterococcus faecium*; *T. pyogenes*: *Trueperella pyogenes*; *E. coli*: *Escherichia coli*; *K. oxytoca*: *Klebsiella oxytoca*; *K. pneumoniae*: *Klebsiella pneumoniae*.

recommended. A questionnaire survey conducted by The Society of Farm Animals in Infectious Disease in Japan in 2014 received responses from approximately 300 veterinarians in 45 prefectures, and 88.7% of veterinarians were performing antimicrobial susceptibility testing for bovine mastitis[14]. Whether this high testing rate is due to the widespread use of the simplified-ADD is unknown, but it is desirable, at least from the perspective of evidence-based antimicrobial treatment. Because the testing rate is high, to use the test results as useful data, determination of appropriate criteria and establishment of a practicable quality control procedure are required.

Now that antimicrobial resistance has become an important international issue, reconsideration of antimicrobial use in the livestock field is also required. To monitor the prevalence of antimicrobial resistance and optimize the use of antimicrobials, carrying out antimicrobial susceptibility tests with the correct procedure with proper quality control and interpreting the results accurately are very important. Because the validity of the simplified-ADD, which is the most popular antimicrobial susceptibility test for bovine mastitis at the clinic level in Japan, was demonstrated in this study, determining the criteria that should be adapted for this method is needed.

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