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Evaluation of the biofilm detection capacity of the Congo Red Agar method for bovine mastitis-causing bacteria

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Abstract

The Congo Red Agar method (CRAM), a method for detecting the presence of bacterial biofilm-forming capacity, does not provide sufficient knowledge on the criteria for each bacterial species. In this study, the biofilm detection capacity of the CRAM and the criteria for determining the presence of biofilm-forming capacity of bovine mastitis-causing bacteria were examined. 149 strains isolated from the milk of dairy cows with clinical mastitis were determined for biofilm-forming capacity using the CRAM. The Calgary Biofilm Device Method was also used as a comparative experiment. The study showed that the suitable medium and incubation time in the CRAM differed for each bacterial species, and the criteria for determining the presence of biofilm-forming capacity in each species could be determined.

Key Words: biofilm, bovine mastitis, Congo Red Agar method

Bovine mastitis is the most common disease of dairy cows, and its effective control is vital because it causes significant losses to dairy farmers. Some bovine mastitis-causing bacteria are capable of forming biofilms, and biofilm formation has been shown to be one of the causes of chronic and recurrent mastitis infections[8,23)]. Biofilms are aggregates composed of micro-organisms and extracellular polymeric substances produced by micro-organisms on solid and liquid surfaces[12,27)]. Extracellular polymeric substances are composed of extracellular polysaccharide (EPS), nucleic acids, proteins, lipids, and other biomolecules, and EPS is said to be an important component of biofilms in many bacteria[10,16)].

Bacteria that form biofilms are known to develop resistance to antimicrobials due to factors such as delayed spread of antimicrobials, increased mutation rates, production of enzymes that degrade antibiotics, presence of dormant bacterial cells with low metabolic activity, and increased growth time in the biofilm inner layer[15)]. Bacterial biofilm formation can cause difficulties in the treatment of mastitis, since bovine mastitis is mainly treated with antimicrobials, but there are few reports of the presence of biofilm-forming capacity and positive rates of bovine mastitis-causing bacteria.

Methods for detecting biofilm-forming capacity include the Tissue Culture Plate method (TCP)[7)], Tube method (TM)[6)], Congo Red Agar method (CRAM)[13)], bioluminescence assay[9)], piezoelectric sensor method[4)], and fluorescence microscopic examination[36)]. In this context, the

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CRAM is a simple qualitative test method, but basic knowledge for each bacterial species is insufficient. It has also been reported that the accuracy of the CRAM is low[14], but its accuracy can be improved by adding or replacing some substances or changing some parameters[21,22]. On the other hand, the Calgary Biofilm Device method (CBDM)[19,34] is a method with a high detection rate of biofilm-forming capacity, but it is complicated and involves many procedures.

The purpose of this study was to easily determine the presence of biofilm-forming capacity of bovine mastitis-causing bacteria and to determine whether biofilms are involved in recurrent or chronic (refractory) cases of bovine mastitis. This would allow for treatment strategies that assume biofilm formation, such as treatment with drug concentrations that exceed the minimal biofilm eradication concentration (MBEC), and would be useful for the effective treatment of refractory cases. In this study, the CRAM and CBDM were compared for bovine mastitis-causing bacteria to investigate the biofilm detection capacity of the CRAM and the biofilm-positive rate of each species. Congo Red Agar (CRA) and Modified Congo Red Agar (MCRA) were used as media in the CRAM. Since the color tone of colonies grown on CRA and MCRA may differ among different bacterial species, the criteria for determining the presence of biofilm-forming capacity in the CRAM were established.

A total of 149 strains isolated from the milk of bovine clinical mastitis cases in eight prefectures in Japan (Hokkaido, Miyagi, Tochigi, Saitama, Aichi, Okayama, Ehime, and Kumamoto) between 2015 and 2017 were tested, Including the following: 20 *Staphylococcus aureus* (*S. aureus*), 14 *Staphylococcus saprophyticus* (*S. saprophyticus*), 13 *Staphylococcus xylosus* (*S. xylosus*), 14 *Streptococcus uberis* (*S. uberis*), 10 *Streptococcus dysgalactiae* (*S. dysgalactiae*), 10 *Streptococcus equinus* (*S. equinus*), 8 *Enterococcus faecium* (*E. faecium*), 20 *Escherichia coli* (*E. coli*), 20 *Klebsiella pneumoniae* (*K. pneumoniae*), 10 *Pseudomonas aeruginosa* (*P. aeruginosa*), and 10 *Trueperella pyogenes* (*T. pyogenes*). Milk samples (10 µl) were individually added to 5% sheep blood agar medium and incubated aerobically at 37 °C for 24 hr according to National Mastitis Council methods[1]. The molecular speciation of the obtained strain was performed via polymerase chain reaction (PCR) and DNA sequencing. Bacterial genomic DNA was isolated using the InstaGene Matrix (Bio-Rad Laboratories Inc., Hercules, CA, USA). The 16S ribosomal RNA (rRNA) genes (rDNA) are highly conserved and universally present across all bacterial species[17,24]. However, the high degree of similarity between closely related species has limited the utility of rDNA sequencing for distinguishing several *Staphylococcus* spp. To differentiate between the closely related *Staphylococcus* spp, a new primer set (16S F1, and 23S R1; see below) was designed based on the 16S–23S rDNA intergenic region, and PCR was performed. The primer sequences were as follows: 16S F1 sense primer, 5′-TTCCCGGGTCTTGTACACAC-3′ and 23S R1 antisense primer, 5′-TTGTAACTCCGTATAGAGTGTCC-3′, for staphylococci; 27F sense primer, 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R antisense primer, 5′-GGTTACCTTGTTACGACTT-3′, for bacteria other than staphylococci[18]. The PCR conditions were as follows: denaturation at 95°C for 30 sec, annealing at 61°C for 30 sec, and extension at 72°C for 2 min and 32 cycles for staphylococci; denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 1 min and 35 cycles for bacteria other than staphylococci. The amplified DNA was subjected to agarose gel electrophoresis. The separated DNA bands were extracted from the gel using the Quantum Prep Freeze 'N Squeeze DNA Gel Extraction Spin Columns (Bio-Rad Laboratories, Inc.) and purified using the QIAquick PCR purification kit (Qiagen N.V., Venlo, The Netherlands). The purified products were directly sequenced using Big Dye Terminator v3.1 (Applied Biosystems, Waltham, MA, USA) on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). Identification was performed by confirming the homology of the obtained DNA sequences with known sequences using the Basic Local Alignment Search Tool

(BLAST)[3].

The CRAM used two mediums, CRA and MCRA. The CRA consists of 0.8 g of Congo Red, 36 g of sucrose, and 1 L of Brain Heart Infusion agar medium. First, Congo Red was dissolved in a small amount of distilled water and sterilized in an autoclave (121 °C for 20 min), then added to autoclaved sucrose and Brain Heart Injection agar medium. 20 ml were dispensed into petri dishes and allowed to dry. The MCRA consists of 0.4 g Congo Red, 10 g glucose, and 1 L of Blood Agar Base-2 medium. Congo Red was first dissolved in a small amount of distilled water and sterilized in an autoclave (121 °C for 20 min) and then added to autoclaved glucose and Blood Agar Base-2 medium. 20 ml were dispensed into petri dishes and allowed to dry. Single colonies obtained in the pre-culture were applied to CRA and MCRA, and the color tone of the colonies was observed after 24 and 48 hr of incubation at 37 °C. The biofilm-forming capacity of the strain was also determined by the CBDM and compared with the results of the CRAM.

For the CBDM method, the recovery neutralization medium was first prepared. 1.0 g of L-histidine, 1.0 g of L-cysteine, and 2.0 g of reduced glutathione were dissolved in 20 ml of sterile distilled water. This neutralizer solution was then passed through a 0.20-μm filter using a syringe and stored at -20 °C. Meanwhile, 1 l of Mueller-Hinton liquid medium with 20.0 g of saponin and 10.0 g of Tween-80 was prepared. To 20 ml of this liquid medium, 500 μl of neutralizer solution were added. Single colonies obtained in the pre-culture were dissolved in tryptic soya liquid medium, turbidity adjusted to McFarland 0.5, and further diluted to 10^4 CFU/150 μl using tryptic soya liquid medium and used in this experiment. MBEC™ Biofilm Assay Plate (Innovotech Inc, Edmonton, Canada) was used for biofilm formation. The adjusted bacterial solution was poured into the reagent reservoir, from which 150 μl per well were dispensed with a multichannel pipette and fitted with a pegged lid. To prevent evaporation, vinyl tape was placed around the plates, and they were set in shakers and incubated for 20 hr at 37 °C with rotation at 110 rpm. A flat-bottomed, 96-well plate with 200 μl of recovery medium dispensed per well was prepared, and the pegs were transferred to this plate. After 30 min of equilibrium, the plate was placed in an ultrasonic washer for 30 min. A stainless-steel bat was floated on the surface of the ultrasonic cleaner, and the plate was placed on top of it so that vibrations caused the biofilm to fall from the peg to the plate. A round-bottomed, 96-well plate with 190 μl of Mueller-Hinton liquid medium dispensed per well was prepared, and 10 μl were taken from the recovery medium that had been sonicated to drop the biofilm and dispensed onto the round-bottomed plate. After 20 hr of incubation at 35 °C, those showing white turbidity were judged to have biofilm-forming capacity. The number of strains that formed biofilms for each bacterial species was divided by the number of strains tested and expressed as a percentage as the biofilm-positivity rate.

The percentage of biofilm-positivity in the CRAM and the percentage of agreement of the results with the CBDM are shown in Table 1. The positive rate of biofilms in the CRAM showed bacterial species with different results depending on the medium and incubation time. *S. aureus*, *S. xylosus*, *S. uberis*, *E. faecium*, *T. pyogenes*, and *P. aeruginosa* showed little difference in the biofilm-positive rate depending on the medium and incubation time, whereas *S. saprophyticus*, *S. dysgalactiae*, *S. equinus*, *E. coli*, and *K. pneumoniae* showed different positive rates depending on the medium and incubation time. The biofilm-positive rate for each bacterial species in the CBDM was 100% for *S. aureus*, 64.3% for *S. saprophyticus*, 76.9% for *S. xylosus*, 64.3% for *S. uberis*, 100% for *S. dysgalactiae*, 100% for *S. equinus*, 100% for *E. faecium*, 100% for *E. coli*, 100% for *K. pneumoniae*, and 10% for *T. pyogenes*. The agreement between the results for biofilm formation capacity in the CRAM and CBDM was as follows: 95.2% after 24 hr of incubation in CRA for *Staphylococcus* spp., 81.4% after 48 hr of incubation in MCRA for *Streptococcus* spp., 100% after 24 hr of incubation in MCRA for *E. faecium* and *T. pyogenes*, and 100% after 48 hr of incubation in CRA for *E. coli* and *K.*

Table 1. Biofilm-positivity rates in the CRAM (CRA or MCRA media) and CBDM

Strains (Number of isolates)	CRA (Percentage agreement of results)[a]		MCRA (Percentage agreement of results)[b]		CBDM
	24hr	48hr	24hr	48hr	-
Staphylococcus aureus (20)	100% (100%)	100 (100%)	100% (100%)	100% (100%)	100%
Staphylococcus saprophyticus (14)	64.3% (85.7%)	100% (64.3%)	85.7% (64.3%)	92.9% (71.4%)	64.3%
Staphylococcus xylosus (13)	76.9% (100%)	100% (76.9%)	100% (76.9%)	100% (76.9%)	76.9%
Streptococcus uberis (14)	92.9% (57.1%)	92.9% (57.1%)	100% (64.3%)	100% (64.3%)	64.3%
Streptococcus dysgalactiae (10)	0% (0%)	10% (10%)	100% (100%)	100% (100%)	100%
Streptococcus equinus (10)	0% (100%)	40% (40%)	0% (0%)	80% (80%)	100%
Enterococcus faecium (8)	87.5% (87.5%)	100% (100%)	100% (100%)	100% (100%)	100%
Trueperella pyogenes (10)	10% (100%)	10% (100%)	10% (100%)	10% (100%)	10%
Escherichia coli (20)	25% (25%)	100% (100)	100% (100%)	100% (100%)	100%
Klebsiella pneumoniae (20)	100% (100%)	100% (100%)	90% (90%)	55% (55%)	100%
Pseudomonas aeruginosa (10)	0% (0%)	0% (0%)	10% (10%)	0% (0%)	100%

[a] Percentage agreement of results between CRAM using CRA media and CBDM.
[b] Percentage agreement of results between CRAM using MCRA media and CBDM.
CRAM: Congo red agar method, CRA: Congo red agar, MCRA: Modified congo red agar, CBDM: Calgary biofilm device method

pneumoniae. All 10 *P. aeruginosa* isolates were biofilm-positive by the CBDM, whereas almost all were negative white colonies by the CRAM. The optimum medium and incubation time for each species in the CRA method was 24 hr in CRA and MCRA for *Staphylococcus* spp., 48 hr in MCRA for *Streptococcus* spp., 24 hr in MCRA for *E. faecium*, 24 hr in CRA and MCRA for *T. pyogenes*, and 48 hr in CRA for *E. coli* and *K. pneumoniae*. Biofilm-forming capacity was identified in the CRAM when the following colonies could be identified: Black and light black for *Staphylococcus* spp., *Streptococcus* spp., *E. faecium*, and *T. pyogenes*, red, black-red, and black for *E. coli* and *K. pneumoniae* (Fig. 1).

There were differences in the coloration of biofilm-positivity in the CRAM for the 10 bacterial species tested in this study, with the exception of *P. aeruginosa* (Fig. 1). The CRAM detects EPS, the main component that forms biofilms[11]. It has been reported that the components of biofilms vary between bacteria species[5,30,31], and it is thought that the differences in the EPS produced by different species of bacteria led to differences in the color tone that was positive by the CRAM.

On the other hand, *P. aeruginosa* strains with biofilm-forming ability showed a negative white color with both CRA and MCRA. Biofilm-forming bacteria break down sucrose in CRAs and glucose in MCRAs as a carbon source, causing bacteria to form EPS. In contrast to other species, *P. aeruginosa* is glucose non-fermentative and does not break down sucrose or glucose, but produces alginate as EPS.[21] Therefore, it is considered that *P. aeruginosa* biofilms formed without the use of sugars could not be detected.

S. dysgalactiae and *S. equinus* showed significant differences in results between CRA and MCRA compared with the CBDM (Table 1). Bacterial strains that were negative for biofilm formation on CRA medium became positive on MCRA medium. It has been noted that incubation in a medium containing glucose at appropriate concentrations promotes biofilm formation[29], and the fact that biofilms did not form in CRAs without glucose, but in MCRAs with glucose, was considered a factor.

In the present study, it was found that many of the bacteria causing bovine mastitis have biofilm-forming capacity (Table 1). Comparison

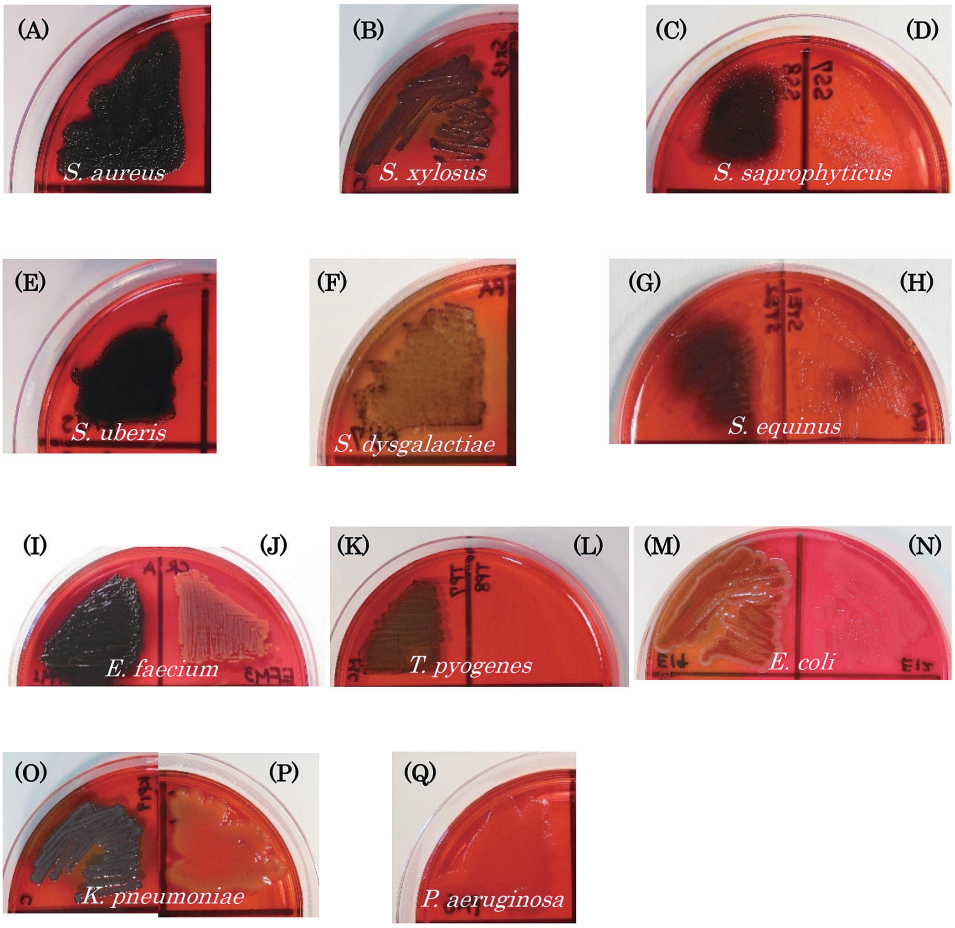

Fig. 1.
In biofilm-positive cases, *S. aureus* (A), *S. xylosus* (B), *S. saprophyticus* (C), *S. uberis* (E), *S. dysgalactiae* (F), *S. equinus* (G), *E. faecium* (I) and *T. pyogenes* (K) showed black or light black colonies. *E. coli* (M) and *K. pneumoniae* (O) showed black, black-red or red colonies.

In biofilm-negative cases, *Staphylococcus* spp. (D), *Streptococcus* spp. (H), *E. faecium* (J), *T. pyogenes* (L), *E. coli* (N), *K. pneumoniae* (P) and *P. aeruginosa* (Q) showed white or colorless colonies.

of previous reports on biofilm-forming capacity with the present study confirmed similar results for biofilm-positivity rates of *S. aureus*, CNS, *S. dysgalactiae*, and *E. coli*[25,28,33]. On the other hand, Sarah et al. (2017)[33] reported positive rates of 100% and 53.1% for *S. uberis* and *Klebsiella* spp. biofilms, respectively, whereas in the present study, the rates were 64.3% for *S. uberis* and 100% for *K. pneumoniae* in the CBDM; these results differed. *K. pneumoniae* has been reported to cause mutations in certain genes that may also affect biofilm function[35]. There are also reports of genes involved in the promotion of biofilm formation in *S. uberis*[32]. It has also been reported that the composition and structure of EPS can vary significantly depending on the type of microorganism and host environment[16]. Therefore, it is possible that differences in genotype, bacterial species, and host environment may have led to differences in the biofilm-positivity rates. In particular, the biofilm-positive rate of *K. pneumoniae* in the present study was 100% in agreement with the CBDM on CRA medium, whereas the agreement decreased on MCRA medium, reaching 55% after 48 hr of incubation, suggesting that differences in medium may have affected the results. The medium used in the study by Sarah et al. (2017) is also consistent with the results of the present study because it contains glucose, like MCRA medium. *T. pyogenes*

was reported to form biofilms in 90% of the tissue culture plate methods[2], which differs significantly from the 10% biofilm-positive rate in the present study. It has been reported that *T. pyogenes* requires the addition of fetal bovine serum and incubation under 10% CO_2 to form biofilms[26], so further studies on culture conditions may be necessary.

The present study demonstrated that the CRAM is suitable for determining the color tone of colonies under the following conditions: after 24 hr of incubation in CRA for *Staphylococcus* spp., after 48 hr of incubation in MCRA for *Streptococcus* spp., after 48 hr of incubation in CRA for *E. coli* and *K. pneumoniae*, after 24 hr of incubation in MCRA for *E. faecium*, after 24 hr of incubation in CRA or MCRA for *T. pyogenes*. The color tone of the colonies at the time of biofilm-positivity in each bacterial species was also evident, and the criteria for biofilm-positivity could be determined. However, it was demonstrated that the CRA method could not be used for *P. aeruginosa*. This study demonstrated that the CRAM is simple and has a high detection rate as a test for detecting biofilm-forming capacity, and the determination of criteria for each bacterial species has made it possible to easily determine the presence of biofilm-forming capacity in clinical practice. Although the biofilm-positive rate was 100% for several species of bacteria, the number of cases examined in this study was limited and does not reflect the results for all of Japan; therefore, it is necessary to continue to examine each case by CRAM in the future.

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