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Pilot clinical trial: propidium monoazide PCR quantifies reduction of the viable bacterial load after antiseptic preparation of canine oral mucosa

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Received February 13, 2023
Accepted April 17, 2023

doi.org/10.2460/ajvr.23.02.0029

OBJECTIVE
A pilot clinical study to evaluate the use of propidium monoazide PCR (PMA-PCR) in quantifying a reduction of bacterial load after antiseptic use on the canine oral mucosa and skin, comparison of quantitative PCR (qPCR) to PMA-PCR, and comparison of patterns seen between PCR methods and bacterial culture.

ANIMALS
Client-owned dogs (n = 10) undergoing general anesthesia and intravenous catheter placement.

PROCEDURES
The oral mucosa and antebrachial skin of each dog underwent swabs for culture, qPCR, and PMA-PCR before and after antiseptic preparation of each site. Reduction in bacterial load between sampling times was evaluated for each quantification method.

RESULTS
All testing methods found a significant decrease in bacterial load from oral mucosa after antiseptic preparation (culture $P = .0020$, qPCR $P = .0039$, PMA-PCR $P = .0039$). PMA-PCR had a significantly greater reduction of bacterial load after preparation than qPCR ($P = .0494$). Only culture detected a significant reduction after preparation of the skin (culture $P = .0039$, qPCR $P = .3125$, PMA-PCR $P = .0703$).

CLINICAL RELEVANCE
PMA-PCR was able to quantify a reduction of bacterial load after antiseptic preparation of the high-bacterial load environment, with a pattern similar to that of culture, and was more specific than qPCR for detecting viable bacterial load. The results of this study support the use of PMA-PCR for antiseptic effectiveness studies performed on a high-bacterial load environment, such as canine oral mucosa.

Studies of antiseptic effectiveness evaluate the reduction of bacterial load and/or reduction of the incidence of surgical site infections. Culture is currently the gold standard for the isolation and quantification of bacteria and offers the unique ability to provide susceptibility testing for antimicrobial selection. However, culture has several limitations such as underestimation of bacterial load due to the assumption that a single organism is responsible for each colony-forming unit, false negatives due to loss of organisms during transport and storage, or false negatives due to the inability of culture to cultivate certain bacteria, including viable but nonculturable (VBNC) organisms.[1] Bacteria may enter into the VBNC state due to adverse environmental conditions including exposure to bacteriostatic or sublethal concentrations of antimicrobials or antiseptics.[2] These VBNC bacteria have an intact cell membrane, metabolic activity, and active transcription and are not dead bacteria.[3] Viable but nonculturable pathogenic bacteria, therefore, present a potential threat to surgical patients, as nearly all are exposed to antiseptics or antimicrobials before the creation of a surgical wound.

Therefore, a need exists for a reliable, highly sensitive, and specific assay for the detection of viable organisms.[2] Molecular techniques such as quantitative PCR (qPCR) have high sensitivity because the process amplifies a single target sequence of DNA. However, this beneficial characteristic extends to

the amplification of DNA from non-viable organisms within the sample, known as "relic DNA." Relic DNA includes DNA that is either extracellular or present in cells that are dead and no longer protected by an intact cell membrane.[2] Relic DNA may be present after antiseptic preparation as many antiseptics destroy the bacterial cell membrane. The presence of relic DNA can lead to overestimation of bacterial load by PCR, miscalculation of the effectiveness of antiseptics, and detection of organisms that are not likely to be clinically significant in the development of surgical site infections.

To address this limitation, propidium monoazide PCR (PMA-PCR), also termed "viability PCR," is a novel technique developed to discriminate between live and dead bacteria; thus, identifying only the viable and VBNC organisms.[4] Before DNA extraction, the sample is pretreated with PMA dye. This dye only penetrates cells that have compromised cell membrane integrity, an accepted biomarker of dead or dying cells.[1] A photolysis step then covalently binds PMA to the DNA, making it inaccessible to amplification by PCR. As antiseptics render bacteria dead by rupturing the cellular membrane while leaving bacterial DNA largely intact, PMA-PCR lends itself to antiseptic effectiveness testing.[5]

In recent years, PMA-PCR has proven clinically useful in its ability to discern between viable and dead bacteria after antiseptic treatment. Abdullah et al (2020) successfully used PMA-PCR to evaluate the efficacy of fluoride solutions and chlorhexidine on naturally grown oral biofilms, proving its usefulness for assessing the effects of antimicrobials in clinical studies.[6] Also in the field of human dentistry, additional studies have proven the ability of PMA-PCR to improve the detection of viable bacteria on dental equipment and the oral cavity after treatment with iodine or chlorhexidine, respectively.[7,8] These studies, along with studies evaluating the use of PMA-PCR with isopropyl alcohol,[9] show promise for PMA-PCR being a feasible option for clinical studies seeking rapid and accurate quantification of viable bacteria, such as with investigations of the effectiveness of antiseptics.

To the best of our knowledge, no study has evaluated the use of PMA-PCR in determining antiseptic effectiveness in the preparation of surgical sites. The objective of this clinical pilot trial was, therefore, to evaluate if PMA-PCR was able to quantify a reduction of bacterial load after antiseptic use on the canine oral mucosa. Secondary objectives included the evaluation of a similar PMA-PCR protocol used for canine skin samples, a comparison of qPCR to PMA-PCR on mucosal and skin samples, and a comparison of patterns seen between PCR methods and bacterial culture.

Materials and Methods

Patient selection

Ten dogs presenting to the Texas A&M University Veterinary Medical Teaching Hospital (College Station, TX) for a planned anesthetic event were sampled. This study was approved by our institution's animal care and use committee (TAMU IACUC 2020-0168 CA) and informed consent was obtained from all owners before the sampling procedure. Dogs requiring IV catheter placement in the cephalic vein and undergoing anesthesia for any indication were eligible for inclusion in the study.

Patient sampling

The fur overlying the cephalic vein was clipped using clean clipper blades in preparation for IV catheter placement. Then, a commercial swab (BD BBL CultureSwab Collection & Transport System, Becton Dickinson) was moistened with a drop of sterile saline and swabbed on the skin over the proposed area of catheter insertion 20 times. Four commercial DNA swabs (Isohelix DNA Buccal Swabs, Cell Projects) were each moistened with a drop of SCF-1 buffer (50 mM Tris buffer [pH 7.6], 1 mM EDTA [pH 8.0], and 0.5% Tween-20) and swabbed on the skin over the same area, 10 times each side. Two swabs were placed into either a PCR-labeled collection tube or a PMA-PCR–labeled collection tube. The catheter site was prepped with commercial antiseptic swabs (ChloraPrep Triple Swabstick, CareFusion) by standard protocol and the sampling procedure was repeated before catheter placement.

After induction of anesthesia, the upper lip of the non-recumbent side was lifted and secured for sample collection. A culture swab and 4 DNA swabs were performed as described above with the exception that the DNA swabs were moistened with sterile saline as opposed to buffer. Sampling was performed at the junction of the buccal and gingival mucosa just dorsal to the upper carnassial tooth. The area was prepped using a commercial antiseptic appropriate for the mouth (Povidone-Iodine Swabsticks, Medline Industries) by standard protocol, and the culture and DNA swabbing procedure was repeated.

Four additional DNA swabs were placed in collection tubes without swabbing to serve as negative controls for both qPCR and PMA-PCR at each DNA extraction event.

Sample handling and preparation

All culture swabs were plated within 24 hours of collection at the same reference diagnostic laboratory (Clinical Microbiology, Texas A&M Veterinary Medical Teaching Hospital). Swabs were plated on 5% bovine blood agar, MacConkey agar, and Columbia CNA Agar with Sheep Blood sequentially, and isolated using a quadrant streak method. Swabs were also inoculated into enriched Tryptose Broth to confirm any potential cases of negative growth on plates. Blood and Columbia CNA plates were incubated at 37 °C in 5% CO_2. MacConkey agar plates were incubated at 37 °C in ambient air. Cultures were examined after 24, 48, and 72 hours of incubation. Semiquantitative scoring of total bacterial growth was based on the highest level of growth on any of the 3 plates. A standard bacterial growth score (BGS) was assigned as follows: growth was recorded 0 for none, 1 for 10 or fewer colonies in the first quadrant

only, 2 for more than 10 colonies in the first quadrant only, 3 for growth spreading into the second quadrant, 4 for growth spreading into the third quadrant, and 5 for growth spreading into the fourth quadrant.

PMA treatment for PMA-PCR samples

For DNA swabs, all PMA treatment, DNA extraction, and qPCR were performed by the same reference diagnostic laboratory (Gastrointestinal Laboratory, Texas A&M Veterinary Medicine & Biomechanical Sciences). The principle of PMA modification of nonviable DNA is shown as a schematic representation **(Figure 1)**. PMA treatment was performed with propidium monoazide dye (PMAxx dye; Biotium) following the manufacturer's instructions.[10] Briefly, 500 uL of sterile PBS was placed into each swab tube and vortexed for 5 minutes. The solution was then transferred to a light-transparent microcentrifuge tube and mixed with a 1:8 dilution of PMAxx working solution to achieve a final concentration of 25 uM (2.5 uL of 2.5 mM working solution added to 250 uL microbial cells). Sample tubes were incubated in the dark for 10 minutes at room temperature with mixing on a rocker. A photoactivation device (PMA-Lite LED Photolysis Device; Biotium) was used to expose the samples to light to allow for cross-linkage of PMAxx to DNA for 15 minutes. All solution samples were added to bead tubes (PowerBead Pro; Qiagen) for DNA isolation.

DNA isolation and quantitative PCR for qPCR and PMA-PCR samples

Genomic DNA isolation was performed using a DNA extraction kit (DNeasy PowerSoil Pro Kit; Qiagen) following the manufacturer's instructions. The qPCR assays for total bacteria on PMA-treated and non-PMA samples were performed using Real-time PCR thermal cyclers (Bio-Rad CFX96 qPCR/Real-Time PCR Module w/C1000 Touch Thermal Cycler system; Bio-Rad Laboratories). Briefly, the concentration of the extracted DNA was measured by a spectrophotometer (NanoDrop 2000; Thermo Scientific) and normalized to 5 ng/µL. A mixture of 2 µL normalized DNA extract (5 ng/µL), 5 µL of SYBR Green supermix (SsoFast EvaGreen supermix; Bio-Rad Laboratories), 0.4 µL of each forward (Universal F341; CCTACGGGAGGCAGCAGT) and reverse (Universal 518R; ATTACCGCGGCTGCTGG) primer (final concentration; 400 nM), and 2.2 µL DNA-free water, in a final reaction volume of 10 µL. The qPCR conditions were as follows: initial denaturation at 98 °C for 2 minutes; 35 cycles of denaturation at 98 °C for 5 seconds; annealing at 59 °C for 5 seconds. All samples were analyzed in duplicate, and the 2 results' average was used for further analysis and data expressed as Log DNA. Real-time PCR analysis software (CFX Maestro 2.3; BioRad Laboratories) was applied to qPCR results.

Statistical analysis

Analysis was performed using statistical graphing software (Prism version 9.3.1; GraphPad Software). Paired data (pre- and post-preparation BGS or total DNA at each sampling site) were compared using the Wilcoxon signed rank test. BGS or total DNA at the different sampling locations (skin or oral mucosa) and the reduction of BGS or total DNA between sampling locations were compared using 2-way ANOVA. A reduction of BGS or total DNA was determined by the difference in the pre- and post-preparation quantification and this unpaired data were compared between the sampling sites with the Mann-Whitney *U* test. Total DNA detected by each PCR method on skin and oral swabs was compared

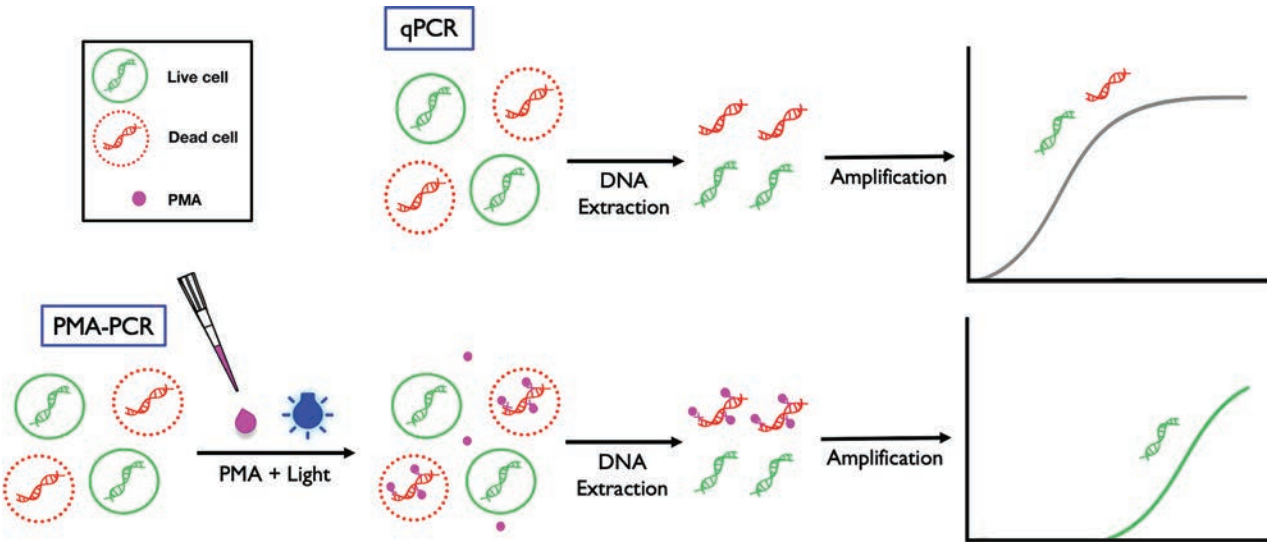

Figure 1—Schematic representation of qPCR and PMA-PCR protocol for processing of DNA samples. After an incubation and photolysis step, PMA covalently binds to DNA within cells with compromised cell membranes, preventing amplification of relic DNA. qPCR = quantitative PCR. PMA-PCR = propidium monoazide PCR. (Adapted from *Viability PCR: rapid & sensitive detection of viable microbes*. Biotium. Accessed April 11, 2023. https://biotium.com/technology/pma-for-viability-pcr/. Reprinted with permission.)

with the negative controls with 2-way ANOVA. The level of significance was set at $P < .05$.

Results

Results of the culture semi-quantification and total DNA detected by qPCR and PMA-PCR are depicted **(Figures 2 and 3)**. The mean, median, and ranges for each quantification method, site, and sampling time, as well as negative controls are listed **(Table 1)**.

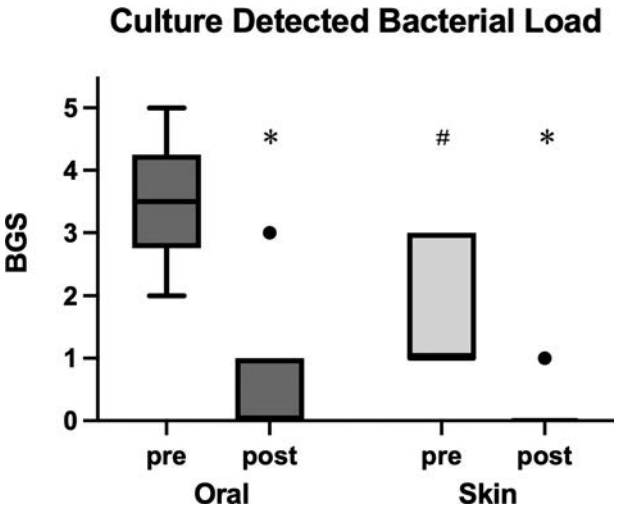

Figure 2—Bacterial load detected by culture on the oral mucosa and skin of 10 client-owned dogs, pre- and post-antiseptic preparation. For each box-and-whisker plot, the solid line within the box represents the median; the lower and upper limits of the box represent the interquartile (25th and 75th percentiles) range, respectively; the whiskers delimit the range; and circles represent outliers. Circles beyond the whiskers indicate outliers. $P < .05$. BGS = bacterial growth score.

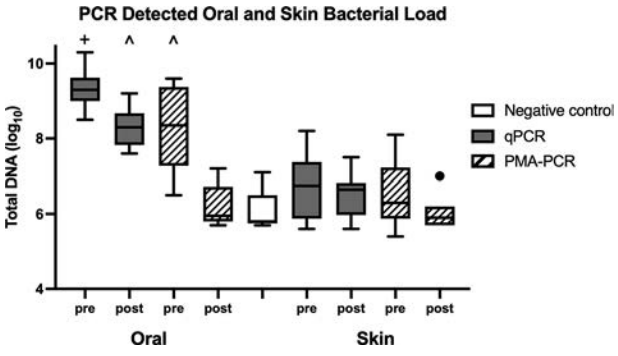

Figure 3—Bacterial load detected by qPCR and PMA-PCR on the oral mucosa and skin of 10 client-owned dogs, pre- and post-antiseptic preparation. For each box-and-whisker plot, the solid line within the box represents the median; the lower and upper limits of the box represent the interquartile (25th and 75th percentiles) range, respectively; the whiskers delimit the range; and circles represent outliers. Circles beyond the whiskers indicate outliers. $P < .05$. qPCR = quantitative PCR. PMA-PCR = propidium monoazide PCR.

Oral samples

For bacterial culture, all pre-preparation oral samples had positive bacterial growth. Six of the 10 post-preparation samples were negative. Antiseptic preparation significantly decreased bacterial growth scores ($P = .0020$).

For qPCR and PMA-PCR, antiseptic preparation significantly decreased total DNA ($P = .0039, .0039$). The reduction of total DNA was significantly different between qPCR and PMA-PCR ($P = .0494$), with PMA-PCR showing a greater reduction. The total DNA detected by qPCR and PMA-PCR was significantly different at both the pre-preparation and post-preparation sampling times ($P = .0043, < .0001$).

Skin samples

For bacterial culture, all pre-preparation skin samples had positive bacterial growth, 2 of which were after broth enrichment. All but 1 post-preparation sample was negative for growth; this single positive sample was after broth enrichment. Antiseptic preparation significantly decreased BGS ($P = .0039$).

For qPCR and PMA-PCR, antiseptic preparation did not significantly decrease total DNA ($P = .3125, .0703$). The reduction of total DNA was not significantly different between qPCR and PMA-PCR ($P = .8672$). There was no difference in total DNA detected by qPCR or PMA-PCR at either pre-preparation or post-preparation sampling times ($P = .8126, .1634$).

Comparison of sampling location

When comparing sampling location (oral mucosa or skin), bacterial growth detected by culture was significantly higher for the oral mucosa at the pre-preparation sampling time ($P = .0003$) but not at post-preparation sampling ($P = .6050$). The reduction of BGS was significantly higher for the oral samples ($P = .0151$).

qPCR and PMA-PCR detected a significantly higher total DNA from the oral mucosa vs the skin pre-preparation ($P = .0001, .0001$). This significant difference was still detected by qPCR post-preparation ($P < .0001$), but not PMA-PCR ($P = .8865$). The reduction of total DNA post-preparation was not significantly different between sampling sites (skin and oral mucosa) with qPCR ($P = .05$). A greater reduction of total DNA from oral mucosa was found with PMA-PCR ($P = .003$).

Comparison of sampling locations with negative controls

Pre-preparation total DNA detected on oral mucosa by either PCR method was significantly different from the negative control (qPCR $P < .0001$, PMA-PCR $P = .0002$). Post-preparation total DNA detected on oral mucosa by qPCR was significantly different from negative control (qPCR $P < .0001$), while total DNA detected by PMA-PCR was not significantly different from negative control, PMA-PCR $P = .9335$).

Pre-preparation total DNA detected on the skin by either PCR method was not significantly different

Table 1—Bacterial load detected on oral mucosa and skin sampling sites on 10 client-owned dogs, pre- and post-antiseptic preparation.

	Oral (n = 10)					
	Pre-preparation			Post-preparation		
Variable	Culture (BGS)	qPCR (DNA log_{10})	PMA-PCR (DNA log_{10})	Culture (BGS)	qPCR (DNA log_{10})	PMA-PCR (DNA log_{10})
Mean	3.5	9.4	8.2	0.6	8.3	6.3
Median	3.5	9.3	8.4	0	8.3	6
Range	(2–5)	(0–3)	(6.5–9.6)	(0–1)	(7.6–9.2)	(5.8–7.2)
	Skin (n = 10)					
	Pre-preparation			Post-preparation		
Variable	Culture (BGS)	qPCR (DNA log_{10})	PMA-PCR (DNA log_{10})	Culture (BGS)	qPCR (DNA log_{10})	PMA-PCR (DNA log_{10})
Mean	1.6	6.8	6.5	0.1	6.5	6
Median	1	6.8	6.3	0	6.7	5.9
Range	(1–3)	(5.6–8.2)	(5.4–8.1)	(0–1)	(5.6–7.5)	(5.7–7.0)
	Negative control (n = 4)					
Variable	(DNA log_{10})					
Mean	6					
Median	5.8					
Range	(5.7–7.1)					

Load detected by bacterial culture is reported as a semi-quantitative bacterial growth score (BGS). Total DNA detected by quantitative PCR (qPCR), propidium monoazide PCR (PMA-PCR), and negative controls are reported as log_{10} values.

from the negative control (qPCR $P = .5849$, PMA-PCR $P = .8647$). Post-preparation total DNA detected on the skin by either PCR method was not significantly different from the negative control (qPCR $P = .4470$, PMA-PCR $P > .9999$).

Discussion

All methods found a significant reduction of bacterial load between pre- and post-preparation of the oral mucosa. Both culture and PMA-PCR detected post-preparation levels similar to that of the skin site post-preparation. This finding is expected, as antiseptic preparation should result in minimal viable bacteria no matter the initial bacterial load. However, qPCR continued to find a significantly higher bacterial load of the oral mucosa vs that of the skin post-preparation, likely due to the amplification of present but non-viable bacterial DNA. Thus, PMA-PCR was more specific than qPCR in detecting viable bacteria in a high-bacterial load environment. This, along with the ability of PMA-PCR to detect and quantify a reduction of bacterial load with a pattern similar to that of bacterial culture, supports the use of PMA-PCR in studies of antiseptic effectiveness in higher bacterial load environments.

Of the 3 quantification methods used, only bacterial culture detected a significant reduction of total DNA on the skin after antiseptic application. Therefore, culture was found to be the best method for detecting and quantifying a reduction of bacterial load in a low-bacterial load environment with the methods used in this study. Neither qPCR nor PMA-PCR detected a significant reduction of total DNA on the skin after antiseptic application. In fact, 3 samples were found to have an increase in total bacterial load. These findings may be explained if the number of bacteria in the samples was at or below the detection limit of our PCR methods. Most PCR techniques create a fluorescent signal that is proportional to the amount of analyte being detected within the sample. However, even negative controls in PCR display a baseline level of fluorescence due to noise from sampling, extraction, and qPCR.[11] While there is no formal definition nor standard method for determining the limit of detection in PCR, it has been proposed that the limit of detection can be described as the lowest concentration level that can be determined as statistically different from the negative control.[12] Analysis in the current study confirmed total DNA of skin swabs was not statistically different from negative controls at both sampling times (pre- and post-preparation), supporting the idea that this low-bacterial load environment was too close to the limit of detection to identify a difference after antiseptic preparation with the utilized PCR methods.

The limit of detection of PMA-PCR could likely be improved by optimization of the current methods used, as previous studies have demonstrated a greater sensitivity for detecting specific and elusive organisms with PMA-PCR when compared with bacterial culture. Askar et al (2019) used PMA-PCR to detect pathogens involved in periprosthetic joint infections, which may be culture negative in up to 40% of cases. The authors of that study found that PMA-PCR had higher sensitivity than culture and higher sensitivity and specificity than qPCR, showing its potential to detect residual viable bacteria before reimplantation.[13] The protocol used for processing

PMA-PCR samples in our study was previously optimized by the TAMU Gastrointestinal Laboratory for use on high biomass samples such as feces and oral swabs. This would explain how PMA-PCR could detect a reduction of bacterial load on the oral mucosa, a relatively high-bacterial load environment, but not the skin. Optimization of the PMA protocol involves determining the appropriate dye concentration and duration of light exposure during the photolysis step for the organisms present within the sample. These optimization steps were not performed in the current study, as our secondary objective was to evaluate if a similar protocol used for the oral mucosa would apply to skin samples. Based on our findings, optimization of the PMA-PCR protocol, as well as the use of primers specific to organisms known to be present in high quantities at the anatomic site of interest (ie, skin), should be considered for future studies looking for increased sensitivity and specificity in detecting viable bacterial load.

To further enhance sensitivity, the use of other molecular techniques combined with PMA may be considered for low-bacterial load environments. Gaoh et al (2022) found that PMA combined with droplet digital PCR was more sensitive than culture in detecting a viable pathogen in pharmaceutical products.[14] Droplet digital PCR is a newer molecular technique that allows for the detection of sequence-specific PCR products in potentially tens of thousands of micro-reactions. This method offers several advantages including increased sensitivity and specificity even in low-bacterial load environments, as well as absolute quantification.[15] Therefore, PMA-droplet digital PCR may be a good option for the detection and absolute quantification of bacterial load reduction in future evaluations of antiseptic effectiveness in low-bacterial load environments.

Additional limitations exist for this study. Swabs for culture, qPCR, and PMA-PCR were all performed separately, meaning that each was drawn over the anatomic site sequentially, therefore, slight variation in bacterial load may exist. Skin swabs were premoistened with SCF-1 buffer to help stabilize the DNA until extraction; this buffer contains a detergent, Tween-20, that may affect bacterial viability and may have skewed the skin PMA-PCR results. While both chlorhexidine and iodine have been used in previous studies of PMA-PCR, the concentration of antiseptics used in the current study has not been validated for their use with PMA.[8–10]

Conclusion

The results of this study support the use of PMA-PCR for antiseptic effectiveness studies performed on a high-bacterial load environment, such as canine oral mucosa. The continued use of bacterial culture is recommended for the evaluation of antiseptics in low-bacterial load environments, such as canine skin. Optimization of the PMA-PCR methods for the site of interest, validation of the antiseptics to be tested, and more sensitive PCR methods should be considered for future evaluations of antiseptic effectiveness, especially for low-bacterial load environments.

Acknowledgments

Supported by the GINN grant at Texas A&M University Department of Small Animal Clinical Sciences.

Alicia K. Nye, DVM, MS for the conception of the study, study design, statistical analysis, and drafting of work. Min-Pyo Hong and So Young Park for sample processing and acquisition of data. Jan Suchodolski, Dr vet med, PhD, and Kelley Thieman-Mankin, DVM, MS, for the conception of the study, study design, revision of content, and final approval of the version to be published.

The authors declare no conflict of interest related to this report.

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