



RESEARCH ARTICLE

Development of Reference Materials Enabling Quantitative Comparability for Six Swine Bacterial Pathogens

Jinyoung Park^{1,2}, Changwoo Park¹, Gyu-Tae Jeon³, Bok-Kyung Ku³, Jae-Won Byun³, Mi-Hye Hwang³, Minkyu Park¹, and Seil Kim^{1,2*}

¹Biometrology Group, Korea Research Institute of Standards and Science, Daejeon 34113, Republic of Korea;

²Department of Precision Measurement, University of Science & Technology, Daejeon 34113, Republic of Korea;

³Animal Disease Diagnostic Division, Animal and Plant Quarantine Agency, Gimcheon, 39660, Republic of Korea

¹ Authors contributed equally to this work.

*Corresponding author: stapler@kriss.re.kr

ARTICLE HISTORY (26-570)

Received: May 15, 2026

Revised: August 16, 2026

Accepted: August 19, 2026

Published online: September 10, 2026

Key words:

Bacterial Pathogens

Droplet digital PCR

Reference materials

ABSTRACT

In this study, reference materials (RMs) were developed to enable quantitative comparability for six major swine bacterial pathogens, including *Actinobacillus pleuropneumoniae*, *Glaesserella parasuis*, *Streptococcus suis*, *Mycoplasma hyopneumoniae*, *Mycoplasma hyorhinis*, and *Salmonella enterica* subsp. *enterica* serovar Typhimurium. These pathogens impose significant health and economic burdens on global swine production through severe respiratory diseases, systemic infections, polyserositis, arthritis, and zoonotic risks, resulting in increased mortality, reduced performance, and substantial treatment and vaccination costs. The optimized primer-probe assay targeted conserved genomic regions for each species to ensure broad detection capabilities across relevant strains using droplet digital PCR (ddPCR). RMs were prepared by combining bacterial genomic DNA with a swine tissue matrix mixture, followed by a comprehensive assessment of homogeneity and stability. The ddPCR-based approach demonstrated high reproducibility and sensitivity for homogeneity and stability assessment of RMs, providing absolute quantification without standard curves. Between-bottle homogeneity showed variations of 6.4-22.9%, representing the relative variation among aliquots observed during homogeneity assessment, while stability assessments confirmed reliability during storage at various temperatures and through multiple freeze-thaw cycles. Each RM was assigned a reference value with well-characterized measurement uncertainty. These RMs support quantitative comparability of analytical results under consistent analytical conditions and provide reliable reference values for evaluating diagnostic assays and standardizing molecular testing. However, further comparative evaluations across different analytical platforms, laboratories, and workflows are required to extend their applicability to diverse diagnostic environments.

To Cite This Article: Park J, Park C, Jeon GT, Ku BK, Byun JW, Hwang MH, Park M and Kim S, 2026. Development of reference materials enabling quantitative comparability for six swine bacterial pathogens. Pak Vet J, 46(9): 2220-2229. <http://dx.doi.org/10.29261/pakvetj/2026.210>

INTRODUCTION

Swine bacterial pathogens represent a significant challenge to global swine production, imposing substantial medical and economic burdens on the industry. Specifically, six major pathogens, including *Actinobacillus pleuropneumoniae* (APP), *Glaesserella parasuis* (GPS), *Streptococcus suis* (SS), *Mycoplasma hyopneumoniae* (MHP), *Mycoplasma hyorhinis* (MHR), and *Salmonella enterica* subsp. *enterica* serovar

Typhimurium (ST), are associated with severe respiratory diseases, systemic infections, and zoonotic risks (Gottschalk and Lacouture *et al.*, 2015; Lee *et al.*, 2016; Vanderwaal and Deen *et al.*, 2018; Dazzi *et al.*, 2020; Westerman and Sheats *et al.*, 2021). APP causes pleuropneumonia and remains economically significant in swine grower/finisher operations (Losinger *et al.*, 2005). GPS, the causative agent of Glässer's disease, is characterized by polyserositis, arthritis, and sepsis, with considerable serotype diversity based on capsular

polysaccharides (Zhu *et al.*, 2024). SS is a re-emerging zoonotic pathogen with substantial case fatality among reported human infections worldwide (Dutkiewicz *et al.*, 2017). MHP, the primary pathogen of enzootic pneumonia, causes tremendous financial losses through treatment and vaccination costs, and mortality from secondary infection (Maes *et al.*, 2018). MHR causes polyserositis and arthritis in post-weaning swine (Clavijo *et al.*, 2017). ST is a major multidrug-resistant serovar of invasive non-typhoidal *Salmonella*, posing particular risk to immunocompromised and pediatric populations (Balasubramanian *et al.*, 2019).

PCR-based diagnostic methods are most frequently used to detect the presence of pathogens by targeting specific genes (Maurischat *et al.*, 2015; Rao *et al.*, 2023). For APP, the *apxIVA* gene, which encodes a species-specific RTX toxin, is a common target; however, genetic variations within this gene can lead to reduced amplification efficiency or false negatives due to primer/probe mismatches (Dreyfus *et al.*, 2004; Slivenecka *et al.*, 2025). GPS diagnostics often target 16S rRNA and *infB* genes, but the highly conserved 16S rRNA can cause cross-reactivity with closely related species, leading to false positives (Turni *et al.*, 2010). In SS detection, target genes include *gdh*, *cps*, and *fbpS*; however, the diversity of serotypes from capsule-related genes makes it difficult to detect (Marois *et al.*, 2004; Savcheniuk *et al.*, 2022). Similarly, MHP diagnosis often targets 16S rRNA and several species-specific genes, including *p36*, *p46*, and putative ABC transporter genes (Sibila *et al.*, 2009). The 16S rRNA gene is commonly used for MHR detection; however, its 16S rRNA's low specificity can cause cross-reactivity with MHP (Roos *et al.*, 2019). ST detection frequently relies on *invA*, *fliA*, and *fliB* genes as specific markers (Radji *et al.*, 2010; Maurischat *et al.*, 2015). Different target genes are used for each pathogen, leading to variability in results (Caron *et al.*, 2000). In particular, difficulties in distinguishing serotypes, cross-reactivity between closely related species, and potential false positives have been widely reported (Heymans *et al.*, 2018; Giménez-Lirola *et al.*, 2019; Schuwerk *et al.*, 2020). Cross-validation with antibody-based diagnostic methods, such as ELISA, is sometimes required for an accurate diagnosis (Xin *et al.*, 2024). Recent advances in diagnostic and analytical technologies, including immunoassay-based platforms, have expanded the capability for sensitive detection and quantitative evaluation of biological targets, emphasizing the need for reliable and standardized measurement approaches (Zhang *et al.*, 2022; Zeng *et al.*, 2025). In parallel, comprehensive evaluation of infectious disease-related biological responses has become increasingly important for understanding disease-associated changes and improving our understanding of disease mechanisms (Jia *et al.*, 2023; Xu *et al.*, 2023).

This study was conducted to address these challenges and to develop primer-probe assays combined with droplet digital PCR (ddPCR) validation methodologies. Conventional quantitative PCR (qPCR) has been widely used for pathogen detection; however, the accuracy of the results can be affected by variations in primer-probe sequences and differences in extraction kits (Tan *et al.*, 2015; Park *et al.*, 2021). Furthermore, previous studies

reported that qPCR has limited sensitivity for bacterial detection, particularly at low concentrations (Persson *et al.*, 2018; Park *et al.*, 2021; Park *et al.*, 2023). The ddPCR method was selected for its ability to provide absolute quantification without requiring a standard curve, as it generates binary positive and negative droplet data that enable precise measurements independent of variations in PCR efficiency (Hindson *et al.*, 2011; Pinheiro *et al.*, 2012; Park *et al.*, 2025). Our internal validation comparing ddPCR quantification with traditional optical-based methods demonstrated exceptional stability and reproducibility with consistently low coefficients of variation, confirming its reliability for developing precise reference materials (RM) (Park *et al.*, 2025). This approach overcomes the limitations of existing primers by identifying and selecting the most efficient primer-probe set for each bacterial target gene, ensuring high sensitivity and specificity.

However, the absence of RMs with reference values for these pathogens limits quantitative comparability across different laboratories and diagnostic platforms, compromising accurate disease surveillance and inter-laboratory data comparison. This study was conducted to develop ddPCR-based RMs enabling quantitative comparability for six major swine bacterial pathogens using optimized primer-probe assays and ddPCR-based quantification. By providing reference values with well-characterized measurement uncertainties, these RMs are expected to serve as reliable standard for diagnostic kit validation and multi-institutional collaborative research, ultimately contributing to more effective disease prevention and control strategies in global swine production.

MATERIALS AND METHODS

Primer and probe design: Genome sequences of six swine bacterial pathogens were retrieved from NCBI for multiple sequence alignment using MAFFT (version 7.526) (Katoh *et al.*, 2005). Conserved regions were identified and used to design primer-probe assays targeting each pathogen, with two candidate assays per pathogen selected based on *in silico* analysis (Table 1).

Bacterial strains: The following reference strains were used for this study: APP (ATCC 27090), GPS (ATCC 19417), SS (NCTC 10446), MHP (ATCC 25934), MHR (ATCC 29052), and ST (ATCC 14028). All six bacterial genomic DNA (gDNA) samples used in this study were obtained from the Animal and Plant Quarantine Agency (APQA, Republic of Korea). The strains were cultured according to standard microbiological procedures, specific to the growth requirements of each organism except MHP and MHR. Since MHP and MHR are difficult to culture, lyophilized MHP and MHR were rehydrated using FRIIS Liquid Medium (Merck, Germany), and 500µL of the rehydrated suspension was used for nucleic acid extraction. All gDNA samples were extracted from pure cultured colonies and rehydrated suspensions using the Maxwell® Blood Kit (Promega, USA) according to the manufacturer's instructions and stored at -20°C until use. The concentration of each gDNA was measured using a Quantus™ Fluorometer (Promega, USA) prior to RM production.

Table 1: Primer and probe sequences of the six swine bacterial pathogens.

RM Code (Bacteria, Target gene)	Assay candidate	Direction	Sequence (5' - 3')	Length (bp)	T _m (°C)	Amplicon size (bp)
111-10-572 (APP, <i>apxIVA</i>)	APP_assay_1*	F	CGA TTA TTC TCG TAC TGA AGC CTT	24	62	220
		P	TAC GGA ATG GAA AGA AGG GCT ATT	24	62	
		R	CCTCTCTTAAACGGGCAATAGTT	23	60.9	
	APP_assay_2	F	TTG GCG GGA TTT AAA TCA AAA CGG	24	62	214
		P	CAA TAA TAT CCT TGC TCA GCT TGG	24	62	
		R	GGC TAT AAA ACG GGT TAA AAC TGA AAT	27	62.1	
111-10-573 (GPS, <i>VanY</i>)	GPS_assay_1*	F	ACA AAT GTC AAG CTA TCC TGC GT	23	60.9	189
		P	CAC CAT TGG GGA ACA GAA ATT GAT	24	62	
		R	CGTTCGGCATTGACTAATAACCA	23	60.9	
	GPS_assay_2	F	GGA GGC TAT TTT CAA CAT TTA ACG A	25	60.9	214
		P	AGT TTA TTG GTC TAG AAC CAT GGC	24	62	
		R	AACTATTTACACCAGCGATATTCTC	25	60.9	
111-10-574 (SS, <i>gdh</i>)	SS_assay_1*	F	ATC GGA CCT TCA CTT GAC GTC	21	61.2	228
		P	CAA CTG GTT ACG GTT TGG TTT ACT T	24	62.5	
		R	TTT GGT CTT TGA AGG ATT TAC CGT TT	26	61.7	
	SS_assay_2	F	CCG CAA CCC ACA TGA AAC AGA	25	61.2	152
		P	TTCTCTACACTTGAGCCTGTTTT	24	60.3	
		R	TGG AAC ACG GAA GCT GAT GAT A	22	60.1	
111-10-575 (MHP, <i>P36</i>)	MHP_assay_1*	F	ATC AAA AAG TTA TCG GTA GTG GAA CT	22	62.1	223
		P	CTA ATT CGG TTC AGG CCT ACG T	23	62.9	
		R	TTC CGG TTA GTT TAG AAT AAG CAC AG	26	61.7	
	MHP_assay_2	F	AGG ACT TGC ATC CGA GTA TGG A	26	61.7	194
		P	CCT GAT TTT GCC GAT GGT AAT GC	22	62.1	
		R	TAA TTC GGA TGT TAT CAG CTA CTA AT	26	62.9	
111-10-576 (MHR, <i>P37</i>)	MHR_assay_1*	F	AGC GCA CAT CCA AAC GCA TAT AAA	24	62	217
		P	ACA CAT AAC AAA TCA GCA ACA AAA CCT	27	62.1	
		R	TGG TTA ACA CTT TTT CTA AAC ACT CCA A	28	62.7	
	MHR_assay_2	F	GAT AAA ACC AAA AAC TTC GAT GAC GT	26	61.7	166
		P	AAC AAT TTA TCT ACT GAC AAT CCT GAA T	28	61.5	
		R	GGA GTT ATT ATA CCA GGA TTA TTT TTT AG	29	62.1	
111-10-577 (ST, <i>DUF4435</i>)	ST_assay_1*	F	TGT CCT GGA TGA ATT TCA GCT TC	23	60.9	155
		P	TAG CGT GGC GAT CAT TTC AGG A	22	62.1	
		R	GGC AAC CTA TGA AGT GAA CGA TA	23	60.9	
	ST_assay_2	F	CGC CAT TGA GGT AAA ACA TCA TG	23	60.9	210
		P	GGA AGA ACC AGT AAA TTA GCT AGA	24	62	
		R	TGG TTG ATC GTG ACG CAT GGA	21	61.2	

*All primer-probe assays_1 was selected to quantify the reference material.

Preparation of matrix mixture: Comprehensive swine tissues DNA was used to mimic complex biological conditions and evaluate potential matrix effects. The Pig Tissue Genomic DNA Panel (PG-015), containing gDNA extracted from 15 different swine tissues, was obtained from commercial sources (Zyagen, USA), with each tissue's stock concentration taken as provided in the manufacturer's certificate of analysis. A matrix mixture was prepared by pooling equal amounts of gDNA from each tissue type (3.3ng/μL final concentration) in the RNA Storage Solution (ThermoFisher, USA).

Production of RMs: RMs for each of the six bacterial pathogens were prepared by spiking the respective bacterial DNA with the matrix mixture. For each pathogen, a predetermined amount of gDNA was added to achieve a target gene concentration within a defined copy number range. The spiked mixtures were thoroughly homogenized to ensure uniform distribution of the target DNA throughout the solution. The homogenized mixtures were then aliquoted into small-volume vials as follows: RMs for APP, MHP, and ST were dispensed at 100μL per vial, and RMs for GPS, SS, and MHR were dispensed at 50μL per vial. All aliquoted RMs were stored at -80°C until further use.

ddPCR reaction conditions: All ddPCR reactions were performed using ddPCR Supermix for Probes (Bio-Rad, USA) in a final reaction volume of 20μL, containing 2× Supermix, primers at a final concentration of 500nM each, probe at a final concentration of 250nM, and 5μL of template DNA. Droplets were generated using the QX200 AutoDG ddPCR System (Bio-Rad), and PCR amplification

was performed on an Applied Biosystems Veriti Thermal Cycler (Thermo Fisher Scientific, USA) under the following conditions: enzyme activation at 95°C for 10min, followed by 40 cycles of denaturation at 94°C for 30sec and annealing/extension at 60°C for 1min, and a final droplet-stabilization step at 98°C for 10min, with a ramp rate of 2°C/s throughout. Droplets were read on the QX200 Droplet Reader (Bio-Rad), and data were analyzed using QuantaSoft software (version 1.7.4, Bio-Rad). A no-template control (NTC, nuclease-free water) was included in every ddPCR run performed throughout this study, including all homogeneity and stability measurements, to confirm the absence of contamination and cross-reactivity; a run was accepted as valid only when the NTC exhibited no positive droplets. In addition, three technical replicates were analyzed per vial in every run.

Homogeneity and stability assessment of RMs:

Homogeneity and stability of the RMs were assessed in accordance with document (ISO Guide 35, 2017). For homogeneity testing, ten randomly selected vials from each of the six swine bacterial pathogen DNA RMs (totaling 60 vials) were analyzed using ddPCR (Park *et al.*, 2021, Park *et al.*, 2025, Park *et al.*, 2026), with three technical replicates per vial (30 ddPCR measurements per RM for homogeneity assessment). The method repeatability for *apxIVA* (APP), *VanY* (GPS), *gdh* (SS), *P36* (MHP), *P37* (MHR), and *DUF4435* (ST) target genes was determined as a relative standard deviation (RSD) from triplicate measurements within each vial. These RSD values were combined across the ten vials as a pooled standard deviation (PSD), which was taken as the homogeneity

measure of each RM and propagated as the Type A component of the uncertainty budget. Consistency among vials was additionally evaluated by examining whether the mean copy number of each vial fell within the reference value and its expanded uncertainty. To evaluate stability, the RMs were subjected to both short- and long-term stability assessments. For short-term stability, the RMs were stored at -20 and -80°C and analyzed one vial per temperature condition was analyzed in technical triplicate on 1, 3, 5, 7, and 14 days. Long-term stability was assessed at -80°C with one vial analyzed in triplicate at 1, 6, and 12 months. Additionally, freeze-thaw stability was evaluated using five independent vials, each subjected to a distinct number of consecutive freeze-thaw cycles and analyzed in technical triplicate. At each storage condition and time point, the RM was considered stable when the measured copy number fell within the reference value and its expanded uncertainty range established above.

Reference values and uncertainty: Measurement uncertainties were established through combined type A and B evaluation methods (Joint Committee for Guides in Metrology, 2012). Type A components included measurement repeatability and between-bottle homogeneity variations, while Type B components included threshold determination variability, partition volume fluctuations, and microbalance precision limitations. For threshold variability assessment, the relative standard deviation (RSD) of copy numbers was determined by analyzing the high-, medium-, and low-threshold settings in the QuantaSoft analysis platform. The partition volume uncertainty was calculated as the standard uncertainty based on a uniform rectangular distribution across a documented range of droplet volumes (Pinheiro *et al.*, 2012; Corbisier *et al.*, 2015; Dong *et al.*, 2015; Bogo *et al.*, 2017; Emslie *et al.*, 2019). The microbalance precision uncertainty was incorporated into the uncertainty budget for gravimetrically prepared serial dilutions.

Combined standard uncertainty was calculated from all Type A and Type B components using the Welch-Satterthwaite equation, and expanded uncertainty was determined at the 95% confidence level. An uncertainty budget analysis was performed to identify the dominant sources of measurement uncertainty. The relative contribution of each component was determined by comparing its squared uncertainty to the squared combined uncertainty, expressed as a percentage. All statistical analyses and graphical representations were performed using R version 4.1.2 with reshape2, ggplot2 packages (Wickham *et al.*, 2007; Wickham *et al.*, 2009; R Core Team, 2020). Homogeneity and stability results are presented as the mean and standard deviation of technical replicates at each vial or time point, together with the expanded uncertainty for comparison against the assigned reference value. For stability assessment, the standard deviation of the technical triplicate measurements at each time point was combined in quadrature with the Type B uncertainty components (threshold setting, partition volume, and balance precision) established during reference-value characterization, and the resulting combined uncertainty was expanded using the same coverage factor (k) applied to the reference value, yielding a time-point-specific expanded uncertainty. Stability was evaluated by comparing this time-point-specific expanded uncertainty interval against the

expanded uncertainty interval of the assigned reference value: a time point was considered consistent with stability when the two intervals overlapped.

RESULTS

Validation of primer-probe assays: Prior to assay validation, the concentration of each bacterial gDNA extract was confirmed by fluorometric quantification: 3.5ng/μL (APP), 3.3ng/μL (GPS), 1.1ng/μL (SS), 0.04ng/μL (MHP), 0.107ng/μL (MHR), and 4.08ng/μL (ST). For each target bacterial pathogen, two candidate primer-probe assays were designed and validated using ddPCR. The validation process included a sensitivity assessment through serial dilutions (10^{-1} to 10^{-6}) and an evaluation of assay specificity. All 12 primer-probe assays (two per pathogen) demonstrated a clear separation between the positive and negative droplet populations across the dilution series. For APP, GPS, SS, MHP, MHR, and ST, the final primer-probe assays were selected based on three primary criteria: (1) the absence of positive signals in no-template controls (NTCs), (2) a clear separation between positive and negative droplet populations, and (3) a minimal number of droplets with intermediate fluorescence intensities between the positive and negative clusters. These selected assays were subsequently used for precise quantification of the RMs and showed linearity across the concentration ranges tested, exhibiting appropriate sensitivity for the reliable detection and quantification of the target genes in each bacterial pathogen.

Quantification of RMs: Reference values for the RMs were determined by calculating the arithmetic mean of copy numbers measured by ddPCR using 1μL from each of ten vials. (Fig. 1, 2). For KRISS 111-10-572 (APP), the copy number of the *apxIVA* gene was 3.1×10^5 copies/μL with a between-bottle homogeneity of 13.9%. The expanded uncertainty was determined to be 1.2×10^5 copies/μL. KRISS 111-10-573 (GPS) exhibited *VanY* gene concentrations of 2.5×10^5 copies/μL with an expanded uncertainty of 7.5×10^4 copies/μL. The between-bottle homogeneity of the sample was 10.6%. For KRISS 111-10-574 (SS), ddPCR analysis revealed that *gdh* was present at 5.6×10^4 copies/μL. The expanded uncertainty was determined to be 3.5×10^4 copies/μL, and a between-bottle homogeneity of 22.9%. The *P36* gene in KRISS 111-10-575 (MHP) was quantified at 1.7×10^4 copies/μL with between-bottle homogeneity of 7.9%, and an expanded uncertainty of 4.1×10^3 copies/μL. Quantification of KRISS 111-10-576 (MHR) determined that the *P37* gene was present at 7.1×10^4 copies/μL. This RM demonstrated the smallest between-bottle homogeneity at 6.4%, with expanded uncertainty of 1.5×10^4 copies/μL. The highest concentration was observed in KRISS 111-10-577 (ST), where *DUF4435* gene reached 4.5×10^5 copies/μL with between-bottle homogeneity of 18.9% and an expanded uncertainty of 2.0×10^5 copies/μL (Table 2). An uncertainty budget analysis was performed to quantify the relative contribution of each component to the combined uncertainty (Table 3). Type A uncertainty was the dominant contributor across all six RMs, ranging from 39.9% (MHR) to 77.9% (ST) of the combined

uncertainty. Among Type B components, threshold determination variability made a substantial contribution (15.3-41.4%), being particularly high in SS. While the droplet volume uncertainty was applied as a uniform Type B component for all RMs, its relative contribution to the total uncertainty budget varied significantly, ranging from (3.2-29.4%), with MHR demonstrating the highest contribution, and microbalance precision contributed less than 1% across all RMs.

Stability assessment of RMs: A stability assessment of the six swine bacterial pathogen RMs was conducted during storage over a 12-month period. The RMs were maintained at temperatures of -20 and -80°C for durations of 1, 3, 5, 7, and 14 days, followed by the measurement of copy

numbers for each target gene. The results demonstrated that the RMs remained stable for up to 14 days under both temperature conditions (Fig. 3). This finding confirms that the RMs can maintain reliable reference values when stored and transported at temperatures of -20°C or lower for a two-week period. Freeze-thaw stability was investigated by subjecting five independent vials to one to five consecutive cycles. The copy numbers remained consistent throughout all five freeze-thaw cycles (Fig. 4). Furthermore, long-term stability was assessed by storing the RMs at -80°C for intervals of 1, 6, and 12 months. Analysis revealed no significant degradation in copy numbers after 12 months of storage at -80°C. These results indicate that the RMs provide dependable reference values when preserved at -80°C for extended periods (Fig. 5).

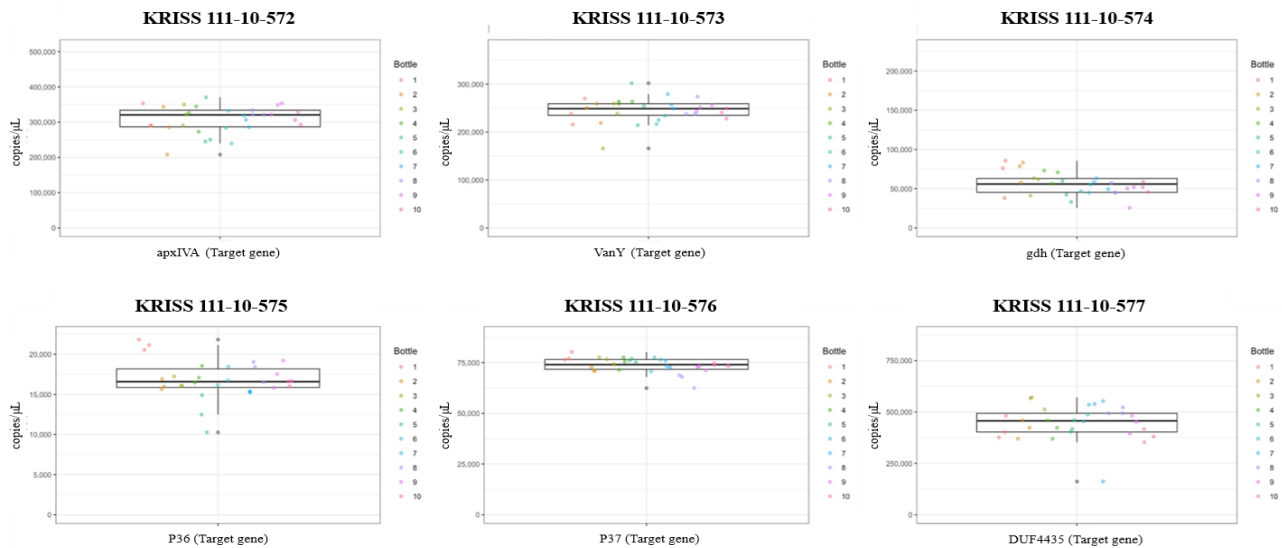


Fig. 1: Droplet digital PCR (ddPCR) quantification of target genes in the KRIS reference materials (RMs). Box plots show the copy number distribution per microliter for six different KRIS RMs (111-10-572 to 111-10-577), based on triplicate measurements from ten vials per RM ($n = 30$ data points per RM). Each plot represents measurements from ten different vials (indicated by different colors) for the following target genes: *apxIVA* (KRIS 111-10-572), *VanY* (KRIS 111-10-573), *gdh* (KRIS 111-10-574), *P36* (KRIS 111-10-575), *P37* (KRIS 111-10-576), and *DUF4435* (KRIS 111-10-577). Box plots illustrate median values, interquartile ranges, and individual data points to demonstrate the between-bottle homogeneity of the reference materials. The copy numbers were determined by averaging the measurements from all ten vials for each reference material.

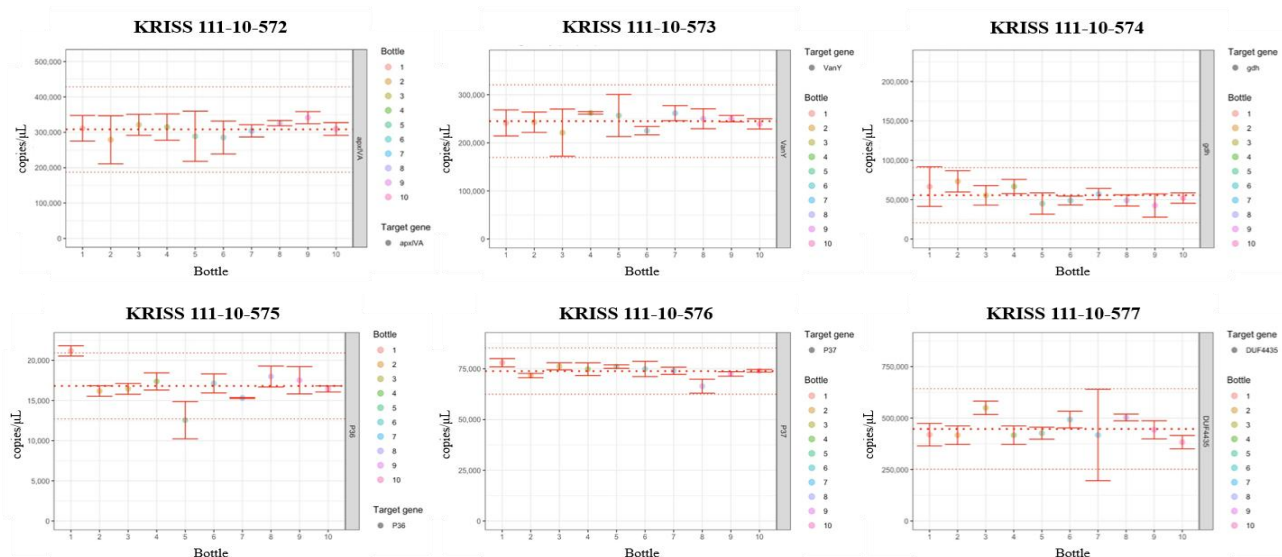


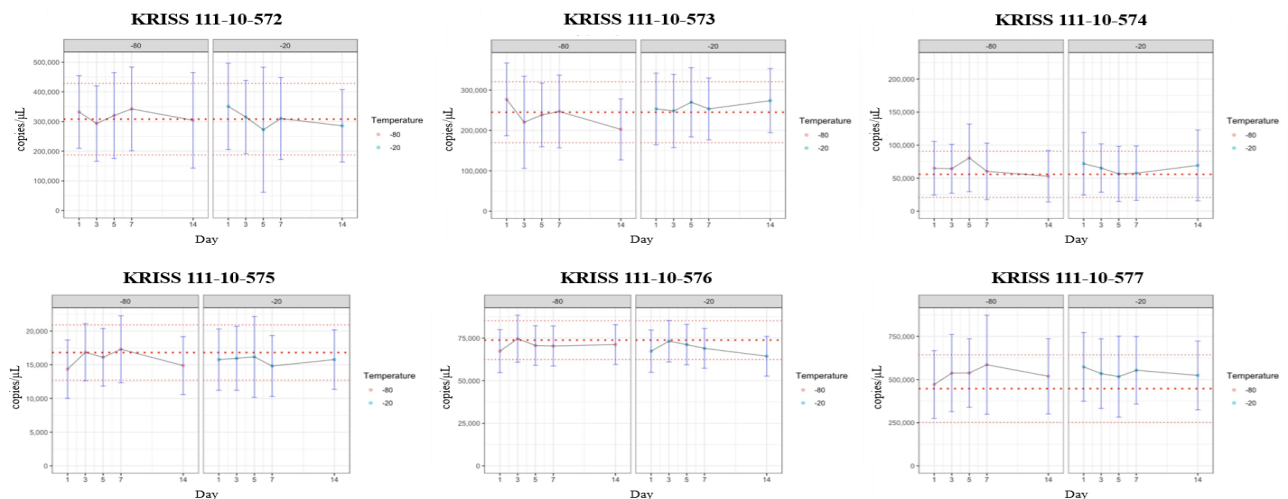
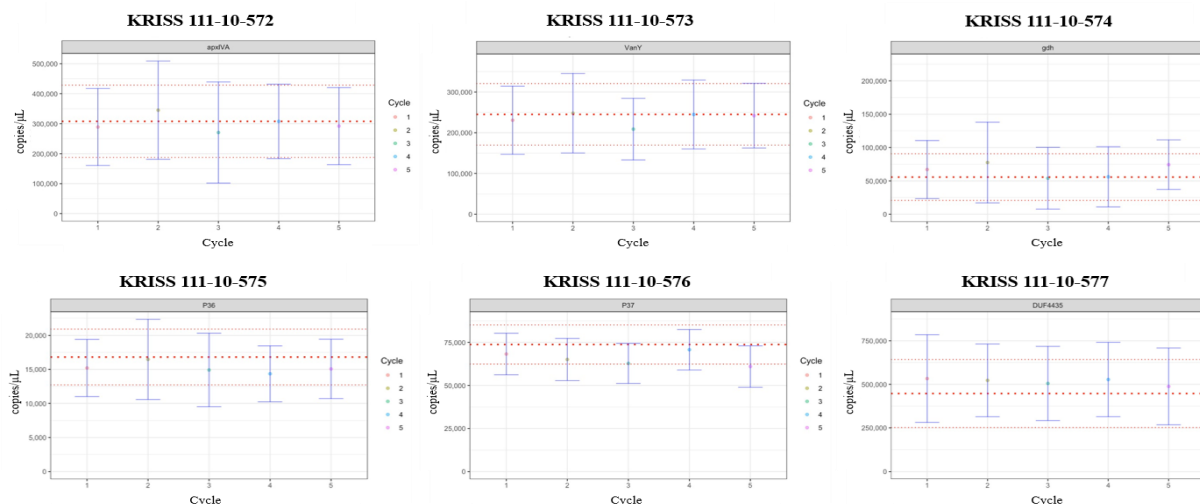
Fig. 2: Homogeneity assessment of RMs showing copy number measurements across ten vials. The graphs display the copy number quantification results (copies/ μ L) for six KRIS RMs (111-10-572 to 111-10-577) across ten vials. Each panel represents a different reference material with its respective target genes: *apxIVA*, *VanY*, *gdh*, *P36*, *P37*, and *DUF4435*. Red points indicate the mean values for each vial with red error bars showing $\pm$ one standard deviation (SD) of the triplicate measurements within each vial. The horizontal red dotted lines represent the reference values (middle line) with their expanded uncertainty bounds (upper and lower dotted lines), demonstrating the consistency and homogeneity of the materials across different vials.

Table 2: Characterization of swine bacterial pathogen RMs.

RM Code	Pathogen	Target gene	Reference value (copies/ μ L)	Homogeneity (%)	Expanded Uncertainty (copies/ μ L)
KRISS 111-10-572	APP	<i>apxIVA</i>	3.1E+05	13.9	1.2E+05
KRISS 111-10-573	GPS	<i>VanY</i>	2.5E+05	10.6	7.5E+04
KRISS 111-10-574	SS	<i>gdh</i>	5.6E+04	22.9	3.5E+04
KRISS 111-10-575	MHP	<i>P36</i>	1.7E+04	7.9	4.1E+03
KRISS 111-10-576	MHR	<i>P37</i>	7.1E+04	6.4	1.5E+04
KRISS 111-10-577	ST	<i>DUF4435</i>	4.5E+05	18.9	2.0E+05

Table 3: Uncertainty budget showing relative contribution of each component contribution.

RM Code	Pathogen	Target gene	Type A		Type B		Total (%)
			Intra-bottle (%)	Threshold (%)	Droplet size (%)	Balance (%)	
KRISS 111-10-572	APP	<i>apxIVA</i>	52.1	39.5	8.1	0.3	100.0
KRISS 111-10-573	GPS	<i>VanY</i>	49.7	36.7	13.1	0.4	100.0
KRISS 111-10-574	SS	<i>gdh</i>	55.3	41.4	3.2	0.1	100.0
KRISS 111-10-575	MHP	<i>P36</i>	43.6	34.7	21.0	0.7	100.0
KRISS 111-10-576	MHR	<i>P37</i>	39.9	29.8	29.4	1.0	100.0
KRISS 111-10-577	ST	<i>DUF4435</i>	77.9	15.3	6.6	0.2	100.0

**Fig. 3:** Short-term stability assessment of KRISS RMs at different storage temperatures. The graphs illustrate the stability of the six KRISS RMs (111-10-572 to 111-10-577) stored at -80 and -20°C for 1, 3, 5, 7, and 14 days. Each panel shows the copy number measurements (copies/ μ L) for the respective target genes (*apxIVA*, *VanY*, *gdh*, *P36*, *P37*, and *DUF4435*) over time. Blue bars represent the expanded combined uncertainty at each time point, derived by combining the standard deviation of the triplicate measurements at that time point with the standard uncertainty of the RM reference value, and expressing the result at the 95% confidence level using the corresponding coverage factor (*k*). The horizontal red dotted lines indicate the reference value (middle line) and the expanded uncertainty limits (upper and lower lines). The consistency of copy numbers across the testing period demonstrates that the RMs maintain reliable reference values when stored at these temperatures for up to two weeks.**Fig. 4:** Freeze-thaw stability assessment of the KRISS RMs. The graphs demonstrate the stability of six KRISS RMs (111-10-572 to 111-10-577) subjected to repeated freeze-thaw cycles (1-5 cycles). Each panel represents copy number measurements (copies/ μ L) for the respective target genes (*apxIVA*, *VanY*, *gdh*, *P36*, *P37*, and *DUF4435*) across five freeze-thaw cycles. Blue bars indicate the expanded combined uncertainty at each cycle, derived by combining the standard deviation of the triplicate measurements at that cycle with the standard uncertainty of the RM reference value, and expressing the result at the 95% confidence level using the corresponding coverage factor (*k*). The horizontal red dotted lines represent the reference value (middle line) and the expanded uncertainty limits (upper and lower lines). The consistency of copy numbers throughout all freeze-thaw cycles confirms that the RMs maintain reliable reference values during multiple freeze-thaw procedures.

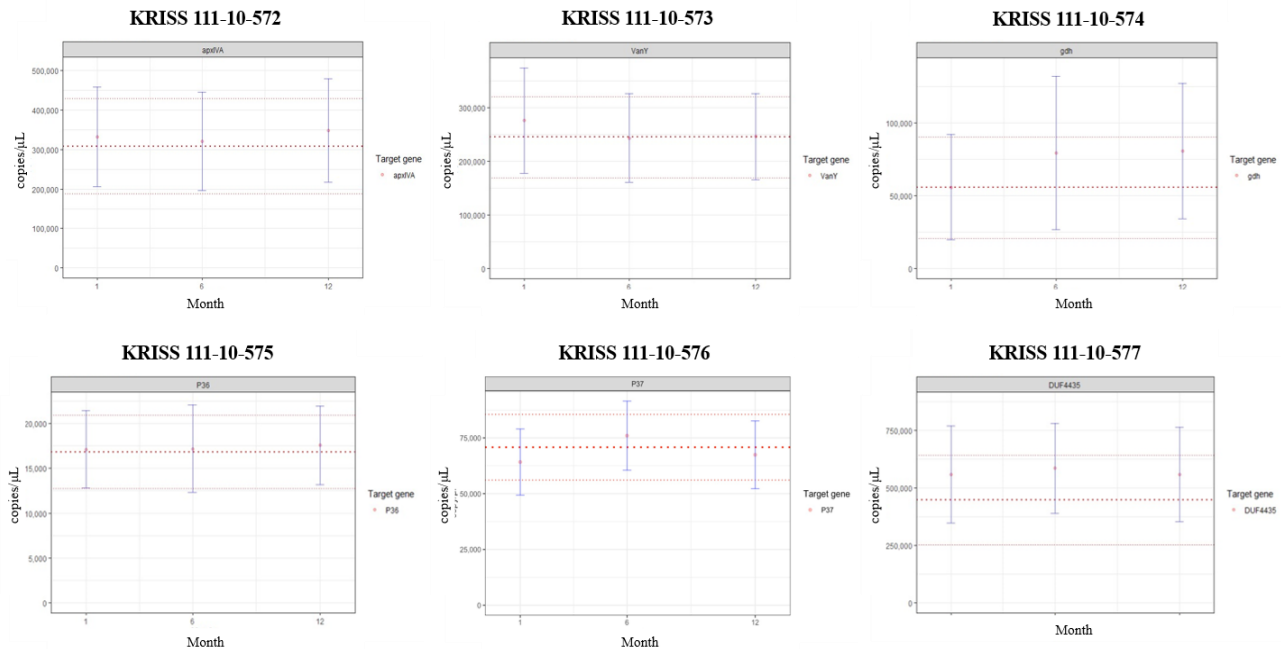


Fig. 5: Long-term stability assessment of KRISS RMs stored at -80°C . The graphs illustrate the long-term stability of six KRISS RMs (111-10-572 to 111-10-577) stored at -80°C over a 12-month period. Each panel shows copy number measurements (copies/ μL) for the respective target genes (*apxIVA*, *VanY*, *gdh*, *P36*, *P37*, and *DUF4435*) after 1, 6, and 12 months of storage. Blue bars represent the expanded combined uncertainty at each time point, derived by combining the standard deviation of the triplicate measurements at that time point with the standard uncertainty of the RM reference value, and expressing the result at the 95% confidence level using the corresponding coverage factor (*k*). The horizontal red dotted lines indicate the reference value (middle line) and its expanded uncertainty limits (upper and lower lines). The consistent copy numbers across the 12-month period demonstrate that the RMs maintained reliable standard values during extended storage at -80°C , confirming their long-term stability for use as reliable quantitative standards.

DISCUSSION

In this study, RMs were successfully developed to enable quantitative comparability for six major bacterial pathogens in swine using ddPCR. This approach addresses the key limitations of conventional qPCR methods by establishing optimized primer-probe assays and validating them through comprehensive homogeneity and stability assessments.

The ddPCR-based approach offers several advantages over the traditional qPCR methods for pathogen detection (Lee *et al.*, 2022; Park *et al.*, 2023). Conventional qPCR techniques frequently produce variable results due to differences in extraction methods, primer-probe sequence variations, and PCR efficiency fluctuations, particularly at low target concentrations (Svec *et al.*, 2015; Park *et al.*, 2021, 2022; Liu *et al.*, 2024). In contrast, ddPCR demonstrated exceptional stability and reproducibility across the concentration ranges tested for all six bacterial pathogens. The binary (positive/negative) nature of droplet analysis eliminates the need for standard curves and reduces the impact of amplification efficiency variations, resulting in more reliable absolute quantification (Lee *et al.*, 2021; Park *et al.*, 2025). This was particularly evident in the consistently low coefficients of variation observed during previous internal validations, which compared favorably with previous studies that reported limitations in qPCR sensitivity for bacterial detection at low concentrations (Persson *et al.*, 2018; Park *et al.*, 2021, 2023; Kim *et al.*, 2023). Furthermore, this approach carefully addresses specific challenges associated with each target pathogen through detailed bioinformatics

analysis and multiple sequence alignments to identify conserved regions, ensuring broad detection capabilities across relevant strains (Park *et al.*, 2025). Although the ddPCR-based quantification approach used in this study is conceptually platform-independent, since droplet partitioning provides absolute quantification without reliance on a standard curve, the reference values reported here were established specifically using the QX200 Droplet Digital PCR System, with primer/probe concentrations and thermal cycling conditions optimized for this instrument. According to a previous cross-platform validation study of ddPCR-based RMs (Lee *et al.*, 2022), comparison of two distinct digital PCR platforms (RT-ddPCR and RT-drPCR) using the same assay and reaction conditions showed measurement values that were generally comparable, with only limited exceptions observed for certain targets. Differences in droplet generation and reading systems, reaction chemistries, and thermal cycling protocols across other ddPCR platforms may affect amplification efficiency and the separation between positive and negative droplet clusters, potentially influencing the measured copy number when these RMs are applied on different instruments. A separate positive control was not included in each run, as every measurement well was itself a characterized positive sample. Assay functionality was therefore confirmed within each run by the clear separation of positive and negative droplet populations obtained, the same validity criterion described by (Cleveland *et al.*, 2024), consistent with practice reported in other ddPCR-based RM studies (Tere-peña and Calderon-ozuna *et al.*, 2024; Falak *et al.*, 2025).

The between-bottle homogeneity values of RMs demonstrated the range from 6.4 to 22.9%. Four of the six RMs (APP, GPS, MHP, MHR; 6.4–13.9%) fall in the similar range of between-bottle homogeneity reported for previous studies (Niu *et al.*, 2023; Lee *et al.*, 2025). Variability of approximately 20% in digital PCR-based quantification has also been reported in a previous study (Tozaki *et al.*, 2021). Although the relatively higher values observed for SS (22.9%) and ST (18.9%) in this study are elevated compared to the other four RMs, these values are considered to remain within an acceptable range for ddPCR-based reference materials, consistent with variability reported in other studies using this technology.

The relatively higher variation observed in SS and ST, may be attributed to the increased viscosity of high-molecular-weight gDNA, which can lead to localized inhomogeneity and pipetting challenges (Koetsier *et al.*, 2021; Michieletto *et al.*, 2022). While DNA shearing techniques could reduce the viscosity and potentially improve homogeneity (Koetsier *et al.*, 2021), intact gDNA was preserved in this study to maintain genomic integrity. The use of intact gDNA ensures that the RMs are more representative of the target pathogens as they are typically encountered in clinical samples. This variation in homogeneity was incorporated into the expanded uncertainty calculations ensuring that RMs provide reliable quantitative standards for pathogen detection despite the inherent physical limitations.

This comprehensive approach to measurement uncertainty combines both Type A and Type B evaluations, thereby providing a robust framework for assessing the reliability of quantitative values assigned to each RM (Lee *et al.*, 2022). The expanded uncertainty, expressed at 95% confidence level, defines intervals within which the true concentration values are expected to lie, enabling end-users to verify agreement with reference values and evaluate the significance of differences in inter-laboratory comparisons. The uncertainty budget analysis provides critical insights into the factors limiting measurement precision. The dominance of Type A uncertainty across all RMs indicates that random measurement variations, rather than systematic factors, represent the primary limitation in precision. These findings indicate that improving measurement repeatability should be the priority for enhancing overall precision in ddPCR-based quantification. The droplet size contribution showed considerable variation, ranging from 3.2 to 29.4%. An important consideration in interpreting the uncertainty budget is that relative contribution percentages reflect proportional relationships rather than absolute magnitudes. The observed variation in droplet size contribution across all RMs is primarily influenced by the magnitude of other uncertainty components rather than changes in the droplet size itself. Although a uniform uncertainty value for droplet size was applied to all RMs based on literature (Pinheiro *et al.*, 2012; Corbisier *et al.*, 2015; Dong *et al.*, 2015; Bogo *et al.*, 2017; Emslie *et al.*, 2019), its proportional weight in the total budget varied. For instance, MHR demonstrated the highest relative contribution from droplet size (29.4%) as a direct consequence of its exceptionally low Type A variation. This suggests that as the precision of the measurement

(Type A) improves, the fixed systematic uncertainties (Type B) inherently become more prominent factors in the overall uncertainty budget of the RMs. This mathematical relationship suggests that, once Type A uncertainty has sufficiently reduced, minimizing Type B factors becomes essential for further precision gains.

The genomic DNA-based RMs developed in this study offer distinct advantages over alternative approaches employed in molecular diagnostics. SARS-CoV-2 packaged RNA reference materials utilize a lentiviral packaging system containing only selected viral genome segments, approximately 12 kilobases encompassing common diagnostic targets such as RdRp, N, E, and S genes (Lee *et al.*, 2022). In contrast, the RMs presented in this study comprise complete bacterial genomic DNA, thereby enabling detection of any genomic region of interest. This whole-genomic coverage provides greater flexibility for laboratories employing diverse target genes or assay platforms. Other nucleic acid RMs for SARS-CoV-2 have employed synthetic DNA fragments, exemplified by 1103-base in vitro transcribed RNA constructs comprising ten discrete target gene fragments (Akyurek *et al.*, 2021). Compared to these synthetic fragments, the whole-genome approach adopted in this study more faithfully recapitulates the complexity and sequence context inherent to authentic pathogen DNA. A similar genomic DNA-based strategy has been applied to bacterial pathogens in a food safety context (Tere-peña and Calderon-ozuna *et al.*, 2024), but not yet to swine bacterial pathogens. Diagnostic assays for swine bacterial pathogens commonly target different genomic regions depending on the manufacturer, so gDNA-based RM offering whole-genome coverage is particularly advantageous for validating kits regardless of the target gene selected.

The RMs developed offer significant practical applications across multiple domains of veterinary diagnostics and disease management. They serve as essential control materials for validating commercial diagnostic kits, enabling laboratories to verify analytical sensitivity, specificity, and quantitative accuracy across various assay platforms. Notably, because each RM was prepared and characterized at a known target copy number, it inherently functions as an internal positive reference, without requiring a separate exogenous positive control in routine use, thereby providing a ready-to-use positive sample for diagnostic assay validation and proficiency testing beyond routine internal quality control. These RMs are particularly valuable for inter-laboratory comparisons, providing common quantitative benchmarks that ensure consistent diagnostic performance and enable meaningful data comparisons between facilities using different methodologies or equipment. Although the genomic DNA-based approach in this study does not include the DNA extraction step, it effectively mimics post-extraction clinical samples by incorporating swine genomic DNA. This strategy replicates the complex biological matrix encountered in real diagnostic workflows and provides a reliable standard for the analytical phase of molecular testing while minimizing pre-analytical variability. The implementation of such RMs can substantially support the standardization and quantitative accuracy of swine disease diagnostics across laboratories and testing platforms.

Conclusions: In this study, RMs were developed and validated to enable quantitative comparability for six major bacterial pathogens relevant to swine health using ddPCR. The developed RMs demonstrated homogeneity with between-bottle variations (ranging from 6.4 to 22.9%) and stability under the transporting and storage conditions, including short-term storage at -20° and -80°C for 14 days, five consecutive freeze-thaw cycles, and long-term storage at -80°C up to 12 months. These characteristics enable the RMs to serve as reliable quantitative benchmarks across laboratories comparisons and over time, with the stability results ensuring integrity during handling conditions and helping to maintain the assigned values.

Enabling absolute quantification without reliance on external standard curves, the ddPCR-based approach can reduce variations associated with amplification efficiency and enhance reproducibility for comparative quantification workflows. The reference values of each RM were established with well-characterized measurement uncertainties, determined through comprehensive Type A and Type B uncertainty evaluations to ensure metrological traceability and reliability. The reported uncertainty enables end users to set acceptance ranges for internal quality control, to define decision thresholds, and to compare results across instruments, kits, and laboratories on a quantitative basis. Uncertainty budget analysis indicated that repeatability was the dominant contributor to the combined uncertainty, suggesting that improving measurement precision would be a practical priority for further reducing uncertainty in future RM production and application.

These RMs can provide reference values for diagnostic validation and enable quantitative comparability across inter-laboratories, which is essential for consistent performance across different platforms in swine pathogen detection. By supporting metrological traceability and quantitative accuracy in ddPCR-based molecular diagnostics, these RMs are expected to contribute to more consistent and comparable pathogen quantification across diagnostic laboratories and testing platforms.

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and materials: All data generated or analyzed during this study are included in this published article.

Declaration of Competing Interest: The authors declare that they have no competing interests.

Funding: This research was supported by grants GP2025-0007-01, and GP2025-0007-04 funded by the Korea Research Institute of Standards and Science. This research was also supported by grants IP2023-0010 and IP2023-0063 funded by the Korea Animal and Plant Quarantine Agency. This research was supported by a National Research Council of Science & Technology (NST) grant from the Korean government (MSIT) (No. GTL24022-

000). This research was supported by the R&D project (Project No. Z-1543069-2023-24-01) on the animal and plant quarantine inspection technology of the Animal and Plant Quarantine Agency in the Republic of Korea.

Acknowledgment: Not applicable.

Declaration of Generative AI and AI-assisted Technologies: Generative AI tool (Claude) was used only for language editing. The authors reviewed and approved all content and remain fully responsible for the final manuscript.

Authors Contribution: SK conceived and designed the study, secured funding, administered and supervised the project. JP and CP executed the experiment, collected, curated and formally analyzed the data, and validated the results, and drafted the manuscript. GTJ, BKK, JWB, MHH and MK executed the experiment, collected and curated the data, and validated the results. SK critically reviewed and edited the manuscript. All authors interpreted the data and approved the final version.

REFERENCES

- Akyurek S, Demirci SNS, Bayrak Z, et al., 2021. The production and characterization of SARS-CoV-2 RNA reference material. *Analytical and Bioanalytical Chemistry* 413:3411–9.
- Balasubramanian R, Im J, Lee JS, et al., 2019. The global burden and epidemiology of invasive non-typhoidal Salmonella infections. *Human Vaccines and Immunotherapeutics* 15:1421–6.
- Bogo A, Divieto C, Pav J, et al., 2017. Droplet volume variability as a critical factor for accuracy of absolute quantification using droplet digital PCR. *Analytical and Bioanalytical Chemistry* 409:6689–97.
- Caron J, Ouardani M and Dea S, 2000. Diagnosis and differentiation of *Mycoplasma hyopneumoniae* and *Mycoplasma hyorhinis* infections in pigs by PCR amplification of the p36 and p46 genes. *Journal of Clinical Microbiology* 38:1390–6.
- Clavijo MJ, Murray D, Oliveira S, et al., 2017. Infection dynamics of *Mycoplasma hyorhinis* in three commercial pig populations. *Veterinary Record* 181:1–7.
- Cleveland MH, He H, Milavec M, et al., 2024. Digital PCR for the characterization of reference materials. *Molecular Aspects of Medicine* 96:101256.
- Corbisier P, Pinheiro L, Mazoua S, et al., 2015. DNA copy number concentration measured by digital and droplet digital quantitative PCR using certified reference materials. *Analytical and Bioanalytical Chemistry* 407:1831–40.
- Dazzi CC, Guizzo JA, Prigol SR, et al., 2020. New pathological lesions developed in Pigs by a “ Non-virulent ” strain of *Glaeserella parasuis*. *Frontiers in Veterinary Science* 7:98.
- Dong L, Meng Y, Sui Z, et al., 2015. Comparison of four digital PCR platforms for accurate quantification of DNA copy number of a certified plasmid DNA reference material. *Scientific Reports* 5:13174.
- Dreyfus A, Schaller A, Nivollet S, et al., 2004. Use of recombinant ApxIV in serodiagnosis of *Actinobacillus pleuropneumoniae* infections, development and prevalidation of the ApxIV ELISA. *Veterinary Microbiology* 99:227–38.
- Dutkiewicz J, Sroka J, Zajac V, et al., 2017. *Streptococcus suis*: A re-emerging pathogen associated with occupational exposure to pigs or pork products. Part I – Epidemiology. *Annals of Agricultural and Environmental Medicine* 24:683–95.
- Emslie KR, McLaughlin JLH, Gri K, et al., 2019. Droplet volume variability and impact on digital PCR copy number concentration measurements. *Analytical Chemistry* 91:4124–4131.
- Falak S, Sullivan DMO, Cleveland MH, et al., 2025. The application of digital PCR as a reference measurement procedure to support the accuracy of quality assurance for infectious disease molecular diagnostic testing. *Clinical Chemistry* 71:378–86.
- Giménez-Lirola LG, Meiroz-De-Souza-Almeida H, Magtoto RL, et al., 2019. Early detection and differential serodiagnosis of *Mycoplasma hyorhinis* and *Mycoplasma hyosynoviae* infections under experimental conditions. *PLoS ONE* 14:1–20.

- Gottschalk M and Lacouture S, 2015. Canada distribution of *Streptococcus suis* (from 2012 to 2014) and *Actinobacillus pleuropneumoniae* (from 2011 to 2014) serotypes isolated from diseased pigs. *The Canadian Veterinary Journal* 56:1093–4.
- Heymans R, Vila A, van Heerwaarden CAM, et al., 2018. Rapid detection and differentiation of *Salmonella* species, *Salmonella Typhimurium* and *Salmonella Enteritidis* by multiplex quantitative PCR. *PLoS ONE* 13:1–15.
- Hindson BJ, Ness KD, Masquelier DA, et al., 2011. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. *Analytical Chemistry* 83:8604–10. ISO Guide 35, 2017.
- Jia Y, Fu B, Dong L, et al., 2023. Sweet syndrome induced by SARS-CoV-2 vaccines: A systematic review of patient-report studies. *Human Vaccines and Immunotherapeutics* 19:2.
- Joint Committee for Guides in Metrology, 2012. International vocabulary of metrology – Basic and general concepts and associated terms (VIM), 3rd edition. Sèvres, France.
- Katoh K, Kuma K, Toh H, et al., 2005. MAFFT version 5: improvement in accuracy of multiple sequence alignment. *Nucleic Acids Research* 33:511–8.
- Kim KW, Lee B, Eom S, et al., 2023. Universal primers for rift valley fever virus whole - genome sequencing. *Scientific Reports* 13:18688:1–11.
- Koetsier PAG, Cantor EJ, Koetsier PAG, et al., 2021. A simple approach for effective shearing and reliable concentration measurement of ultra- high-molecular-weight DNA. *BioTechniques* 71:439–44.
- Lee DH, Ju HJ, Lee Y, et al., 2025. Development of RNA reference materials for norovirus GI and GII using digital PCR. *Virology* 603:110358.
- Lee Jung-ah, Oh Y, Hwang M, et al., 2016. *Mycoplasma hyorhinis* is a potential pathogen of porcine respiratory disease complex that aggravates pneumonia caused by porcine reproductive and respiratory syndrome virus. *Veterinary Immunology and Immunopathology* 177:48–51.
- Lee SS, Park JH and Bae YK, 2021. Comparison of two digital PCR methods for EGFR DNA and SARS-CoV-2 RNA quantification. *Clinica Chimica Acta* 521:9–18.
- Lee SS, Kim S, Yoo HM, et al., 2022. Development of SARS-CoV-2 packaged RNA reference material for nucleic acid testing. *Analytical and Bioanalytical Chemistry* 414:1773–85.
- Liu S, Chai X, Liu C, et al., 2024. Sensitivity analysis of RT-qPCR and RT-ddPCR for SARS-CoV-2 detection with mutations on NI and E primer-probe region. *Microbiology Spectrum* 12:1–8.
- Losinger WC, 2005. Economic impacts of reduced pork production associated with the diagnosis of *Actinobacillus pleuropneumoniae* on grower / finisher swine operations in the United States. *Preventive Veterinary Medicine* 68:181–93.
- Maes D, Sibila M, Kuhnert P, et al., 2018. Update on *Mycoplasma hyopneumoniae* infections in pigs: Knowledge gaps for improved disease control. *Transboundary and Emerging Diseases* 65:110–24.
- Marois C, Bougeard S, Gottschalk M, et al., 2004. Multiplex PCR assay for detection of *Streptococcus suis* species and serotypes 2 and 1/2 in tonsils of live and dead pigs. *Journal of Clinical Microbiology* 42:3169–75.
- Maurischat S, Baumann B, Martin A, et al., 2015. Rapid detection and specific differentiation of *Salmonella enterica* subsp. *Enterica* Enteritidis, Typhimurium and its monophasic variant 4,[5],12:i: By real-time multiplex PCR. *International Journal of Food Microbiology* 193:8–14.
- Michieletto D, Neill P, Weir S, et al., 2022. Topological digestion drives time-varying rheology of entangled DNA fluids. *Nature Communications* 13:1–13.
- Niu C, Dong L, Zhang J, et al., 2023. Reference material development for detection of human respiratory syncytial virus using digital PCR. *Analytical and Bioanalytical Chemistry* 415:3131–5.
- Park C, Lee Jina, Hassan ZU, et al., 2021. Comparison of digital PCR and quantitative PCR with various SARS-CoV-2 Primer-Probe Sets. *Journal of Microbiology and Biotechnology* 31:358–67.
- Park C, Kim KW, Park D, et al., 2022. Rapid and sensitive amplicon-based genome sequencing of SARS-CoV-2. *Frontiers in Microbiology* 13:876085.
- Park C, Park D, Hassan ZU, et al., 2023. Comparison of RT-qPCR and RT-ddPCR with Rift valley fever virus (RVFV) RNA. *Scientific Reports* 13:3085.
- Park C, Park J, Chang D, et al., 2025. Development of reference-based model for improved analysis of bacterial community. *Food Research International* 211:116380.
- Park M, Park C, Kim SB, et al., 2026. Optimized viability-ddPCR with Triton X-100 enhancement for selective detection of live *Salmonella enterica* and *Cronobacter sakazakii*. *Journal of Microbiology and Biotechnology* 36:1–10.
- Persson S, Eriksson R, Lowther J, et al., 2018. Comparison between RT droplet digital PCR and RT real-time PCR for quantification of noroviruses in oysters. *International Journal of Food Microbiology* 284:73–83.
- Pinheiro LB, Coleman VA, Hindson CM, et al., 2012. Evaluation of a droplet digital polymerase chain reaction format for DNA copy number quantification. *Analytical Chemistry* 84:1003–11.
- R Core Team, 2020. The R Project for Statistical Computing.
- Radji M., Malik A. and Widyasmara A., 2010. Rapid detection of *Salmonella* in food and beverage samples by polymerase chain reaction. *Malaysian Journal of Microbiology* 6:166–70.
- Rao J, Wei X, Li H, et al., 2023. Novel Multiplex PCR Assay and Its Application in Detecting Prevalence and Antibiotic Susceptibility of Porcine Respiratory Bacterial Pathogens in Guangxi, China. *Microbiology Spectrum* 11:e03971-22.
- Roos LR, Nair MS, Rendahl AK, et al., 2019. *Mycoplasma hyorhinis* and *Mycoplasma hyosynoviae* dual detection patterns in dams and piglets. *PLoS ONE* 14:1–12.
- Savcheniuk MO, Tarasov OA, Zakharova OM, et al., 2022. Detection of *Streptococcus suis* using the optimized real-time polymerase chain reaction protocol. *Regulatory Mechanisms in Biosystems* 13:168–73.
- Schuwerk L, Hoeltig D, Waldmann KH, et al., 2020. Serotyping and pathotyping of *Glaesserella parasuis* isolated 2012–2019 in Germany comparing different PCR-based methods. *Veterinary Research* 51:1–14.
- Sibila M, Pieters M, Molitor T, et al., 2009. Current perspectives on the diagnosis and epidemiology of *Mycoplasma hyopneumoniae* infection. *Veterinary Journal* 181:221–31.
- Slivenecka E, Jurnecka D, Holubova J, et al., 2025. The *Actinobacillus pleuropneumoniae* apxIV operon encodes an antibacterial toxin-immunity pair. *Microbiological Research* 292:128043.
- Svec D, Tichopad A, Novosadova V, et al., 2015. How good is a PCR efficiency estimate: recommendations for precise and robust qPCR efficiency assessments. *Biomolecular Detection and Quantification* 3:9–16.
- Tan GW, Khoo ASB and Tan LP, 2015. Evaluation of extraction kits and RT-qPCR systems adapted to high-throughput platform for circulating miRNAs. *Scientific Reports* 5:1–8.
- Tere-peña CP and Calderon-ozuna MN, 2024. Digital PCR Validation for Characterization of Quantitative Reference Material of *Escherichia coli* O157: H7 Genomic DNA. *Methods and Protocols* 7:94.
- Tozaki T, Ohnuma A, Kikuchi M, et al., 2021. Design and storage stability of reference materials for microfluidic quantitative PCR-based equine gene doping tests. *Journal of Equine Science* 32:125–34.
- Turni C, Pyke M and Blackall PJ, 2010. Validation of a real-time PCR for *Haemophilus parasuis*. *Journal of Applied Microbiology* 108:1323–31.
- Vanderwaal K and Deen J, 2018. Global trends in infectious diseases of swine. *Proceedings of the National Academy of Sciences* 115:11495–500.
- Westerman TL and Sheats MK, 2021. Sulfate import in *Salmonella Typhimurium* impacts bacterial aggregation and the respiratory burst in human neutrophils. *Infection and Immunity* 89:6.
- Wickham H, 2007. Reshaping data with the reshape package. *Journal of Statistical Software* 21:1–20.
- Wickham H, 2009. ggplot2: Elegant Graphics for Data Analysis. Use R!
- Xin L, Wang H, Hu Y, et al., 2024. The establishment and application of a one-step multiplex real-time polymerase chain reaction assay for the detection of *Streptococcus suis*, 1 serotype 2, and *Glaesserella parasuis*. *Animal Research and One Health* 2:59–70.
- Xu K, Gao B, Li J, et al., 2023. Clinical features, diagnosis, and management of COVID-19 vaccine-associated Vogt-Koyanagi-Harada disease. *Human Vaccines and Immunotherapeutics* 19:2. (qPCR) for enumeration of *Cryptosporidium* oocysts in faecal samples. *International Journal for Parasitology* 44:1105–13.
- Zeng H, Zhang Y, Zhang M, et al., 2025. A signal enhanced lateral flow immunoassay based on core-shell quantum dots labeled antibody and antigen for sensitive detection of CP4-EPSPS protein. *Microchemical Journal* 209:112758.
- Zhang J, He M, Xie Q, et al., 2022. Predicting in vitro and in vivo anti-SARS-CoV-2 activities of antivirals by intracellular bioavailability and biochemical activity. *ACS Omega* 7:45023–35.
- Zhu Y, Guan L, Zhang J, et al., 2024. Research progress into the principles and methods underlying capsular typing of *Glaesserella parasuis*. *Veterinary Research* 1–12.