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Rapid Colorimetric Point-of-Care Detection of Live Coliform Bacteria Using PMA-LAMP-mAuNP with Method Validation

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Abstract

Coliform bacteria are ubiquitous in environments, including in the feces of humans and animals, and comprise diverse species. Their presence in drinking water and food products is prohibited (generally limited to ≤10 coliform counts/g or mL). Traditional culture methods detect viable coliforms but require 5-7 days of cultivation, whereas conventional PCR/qPCR assays shorten detection time (~4–5 h) but are costly (~60–100 USD per species per reaction), are unable to discriminate between live and dead cells, and cannot simultaneously detect multiple coliform genera in a single reaction. This study thereby developed a rapid, multiplex, colorimetric assay for viable coliform detection using propidium monoazide–loop-mediated isothermal amplification with multiplex nanogold probes (PMA-LAMP-mAuNP). The assay enables simultaneous detection of multiple live coliform species within 80 minutes (40 min viable genetic extraction, 40 min genetic amplification-detection) with a limit of detection of 1 colony forming unit (CFU), is low-cost (~4-5 USD/reaction), and requires only a portable temperature controller and a simple halogen light source, making it field- and point-of-care–ready. Our PMA-LAMP-mAuNP demonstrated high specificity and sensitivity across live and dead cells, pure and mixed cultures of *Escherichia*, *Shigella*, *Klebsiella*, *Enterobacter* and *Salmonella*, as well as against non-coliform species. Visual color readouts allowed quantitative estimation of coliform concentrations with reliable linear regressions (R^2=0.9665-0.9804). Furthermore, the assay achieved 100% sensitivity for total bacteria and *Escherichia coli* (coliform) detection, comparable to the reference culture method (90.91% sensitivity), with consistent relative trueness, relative limits of detection, and zero false positives. Hence, this PMA-LAMP-mAuNP assay is a validated and field-deployable platform for rapid, multiplexed detection of viable coliform bacteria at the point-of-care.

Keywords: Loop-Mediated Isothermal Amplification (LAMP); Nanogold Probe (AuNP); Coliform; Rapid Detection; Bacteria; Propidium Monoazide (PMA); ISO 16140 Method Validation.

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1. Introduction

Coliform bacteria are gram-negative, non-spore-forming bacilli commonly used as indicators of sanitary quality in water and food products. Their presence often reflects fecal contamination and potential health risks from species such as *Escherichia*, *Klebsiella*, *Salmonella*, *Enterobacter*, and *Shigella*) [1-3]. Coliform-related infections can cause vomiting, bloody diarrhea, stomach cramps, and fever. The World Health Organization (WHO) and the U.S. Food and Drug Administration (FDA) stipulate that coliforms should be absent or ≤ 10 counts per gram or milliliter in drinking water and grade "A" dairy products [4-6]. Contamination of water resources with coliforms remains a leading cause of child mortality in low-income and developing countries [3, 4].

Traditional culture-based methods employ a variety of media—including violet red bile agar, sorbitol MacConkey agar, lauryl sulfate tryptose broth, and brilliant green bile broth—followed by multiple steps of sample enrichment, selective culturing, and biochemical confirmation. These workflows require 5–7 days to complete and rely on trained microbiologists [4, 5, 7]. Moreover, culture-based assays can show low sensitivity in certain food matrices such as pork or meat [4, 8, 9].

Molecular methods such as conventional PCR and quantitative real-time PCR (qPCR) have improved detection speed (~4–5 h) and sensitivity but remain constrained by high cost, the need for sophisticated laboratory equipment, and the inability to distinguish live from dead cells or to simultaneously detect multiple coliform genera within a single reaction [9-12]. PCR followed by gel electrophoresis (PCR-GE) or qPCR still requires ~4-5 h per assay and is relatively expensive (~60-100 USD/species/reaction) [4, 10, 11]. Subsequently, both culture-based and PCR-based methods are unsuitable for field deployment or point-of-care testing, as they require sample transportation to centralized facilities, further increasing turnaround time and cost.

Several studies and the WHO have reported the use of loop-mediated isothermal amplification (LAMP) as a promising alternative for pathogen detection in field and point-of-care settings [12-18]. LAMP operates under isothermal conditions, tolerates common inhibitors, and eliminates the need for costly thermal cyclers or specialized DNA extraction kits. This is enabled by *Bst* DNA polymerase with intrinsic strand-displacement activity and loop-forming primers, which together allow robust amplification even under challenging sample conditions (e.g., high salinity or pH) that typically inhibit *Taq* polymerase in PCR [9, 15-17]. However, most existing LAMP assays for coliforms are designed for single-species detection, lack viability discrimination, and have not been validated under the International Organization for Standardization (ISO) 16140 framework, specifically ISO 16140-4 for single-laboratory validation [19-22].

Recent innovations combining propidium monoazide (PMA) with molecular amplification techniques (e.g., LAMP or PCR) have enabled selective amplification of DNA from viable cells of *Escherichia*, *Shigella*, *Klebsiella*, *Enterobacter* and *Salmonella*. PMA penetrates dead cells through compromised membranes and binds DNA, preventing its amplification, thereby ensuring that only live-cell DNA is detected [22-26]. Nonetheless, PMA efficiency varies depending on sample composition and bacterial membrane properties, making optimization essential [23-28]. In parallel, nanogold-based colorimetric probes have been explored for simple, instrument-free visual detection [29-31].

Despite these advances, an integrated, inexpensive, multiplex LAMP platform capable of simultaneously detecting diverse live coliform genera with direct, colorimetric readout in field or point-of-care settings remains unavailable. In contrast, previous nucleic acid–based diagnostics such as real-time PCR involve multiple steps, costly instrumentation, and long assay times, limiting their use outside laboratory environments. Therefore, this study aims to fill this gap by developing a rapid, multiplexed, colorimetric, point-of-care assay for viable coliform detection that integrates PMA-based viability selection with LAMP amplification and multiplex nanogold probes (PMA-LAMP-mAuNP) (see Graphical Abstract for workflow). The mAuNP are designed to specifically hybridize with coliform sequences, eliminating false positives, enhancing assay specificity, and enabling naked-eye color differentiation between positive (red) and negative (purple) samples upon nanoparticle aggregation [9, 31]. The method was further evaluated and validated in accordance with ISO 16140-4 standards against the FDA's Bacteriological Analytical Manual (BAM) reference method to confirm analytical performance [32-34]. Additionally, the visual color readout from LAMP-mAuNP allowed semi-quantitative estimation of viable coliform concentrations [9].

2. Methods

2.1. Preparation of Bacterial Strains

Bacterial strains used in this study included 5 positive (*Escherichia coli*, *Shigella* sp., *Klebsiella pneumoniae*, *Enterobacter aerogenes* and *Salmonella typhimurium*) and 4 negative (*Staphylococcus aureus*, *Bacillus cereus*, *Enterococcus faecalis* and *Vibrio parahaemolyticus*) references (Table 1). These bacterial genera were used to evaluate sensitivity and specificity of the coliform PMA-LAMP-mAuNP assay, and could be found in drinking or food products and/or contaminated from environments. All bacterial strains were propagated in nutrient broth (HiMedia, Mumbai, India) with constant shaking of 180 rpm at 37°C. An overnight culture was transferred to a new fresh nutrient broth and

continuously propagated for 3-5 h. Then, the bacterial cell pellets were collected via centrifugation at 8,000 × *g* for 10 min, resuspended in sterile distilled water, and enumerated for number of cells (colony forming units per mL, CFU/mL) by plate count technique on a nutrient agar (HiMedia).

Table 1. List of bacteria strains used as positive and negative references, and the specificity to each of nanogold probes

Species	Strains	AuNP probes			Sources and/or references
		1	2	3	
Escherichia coli	ATCC25922	+	-	-	Deng et al. [35]
Shigella sp.	MSCU0887	+	-	-	Choopara et al. [15]
Klebsiella pneumoniae	ATCC13883	-	+	-	Supabowornsathit et al. [36]
Enterobacter aerogenes	MSCU0357	-	-	+	This study
Salmonella typhimurium	MSCU0492	-	-	+	This study
Staphylococcus aureus	ATCC25923	-	-	-	Deng et al. [35]
Bacillus cereus	MSCU0389	-	-	-	This study
Enterococcus faecalis	MSCU0494	-	-	-	This study
Vibrio parahaemolyticus	MSCU0646	-	-	-	This study

Note: These strains were provided by the Department of Medical Sciences, Ministry of Public Health; Phramongkutklao Hospital; Department of Microbiology, Faculty of Science, Chulalongkorn University; and Faculty of Medicine, Chulalongkorn University [15, 35, 36].

2.2. Design of LAMP Primers and Coliform mAuNP Probes

The 16S rRNA gene is an accepted universal gene for bacterial species detection with the variable region that differs among species; hence, this gene is worldwide used for bacterial diversity and microbiota analyses [29-31]. Consistently, our LAMP primers were designed based on the universal bacterial species region, and the mAuAP probes (16S-thiol probe 1, 16S-thiol probe 2, and 16S-thiol probe 3) were designed based on ClustalW multiple sequence alignment (www.megasoftware.net/) of coliform bacteria species 16S rRNA gene variable region V3 sequences downloaded from GenBank (www.ncbi.nlm.nih.gov/genbank/) that were unique to coliform bacteria (genera *Escherichia*, *Shigella*, *Klebsiella*, *Enterobacter* and *Salmonella*) against non-coliform bacteria species (Table 2). The variable region V3 was selected because comparative analyses showed high degree of sequence divergence among closely related Enterobacteriaceae species provides strong discriminatory power [37-39], enabling the design of genus-specific probes, such as those for *Escherichia*/*Shigella*. Each mAuNP probe sequence was subsequently verified for specificity to its corresponding coliform species by BLASTN vs. non-redundant database.

Table 2. Coliform LAMP primer and mAuNP probe sequences

Primer name	Sequence (5′ 3′) →
16S-FIP	TAGGAGTYTGGGCCGTGTCTCATTTTACCAAGGCGACGATSSMTA
16S-BIP	CTAACTMCGTGCCAGCAGCCTTTTCCARTAATTCCGANYAACGC
16S-LF	GRTCAYCCTCTCAGACCRGC
16S-LB	GGTAATACGKAGGGKGCRA
16S-F3	ATTAGSTAGTAGGTGRGGT
16S-B3	GCCTRCGCRCGCTTTACG
16S-thiol probe 1	SH-AAAAAAAAAAAGTAAAGTTAATACCTTTGCTC
16S-thiol probe 2	SH-AAAAAAAAAAGTTAAGGTTAATAACCTTGGCG
16S-thiol probe 3	SH-AAAAAAAAAAATTGTGGTTAATAACCACAGTG

Note: LAMP primers are specific to universal bacteria and mAuNP probes are specific to coliform bacteria, respectively. The 16S-thiol probe 1 is specific to coliform genera Escherichia and Shigella; 16S-thiol probe 2, Klebsiella; and 16S-thiol probe 3, Enterobacter and Salmonella.

2.3. LAMP Assay

The LAMP reaction (25 µL) comprised 1.6 µM each of primers 16S-FIP, 16S-BIP, 16S-LF and 16S-LB, 0.2 µM primers 16S-F3 and 16S-B3, 1.4 mM dNTP (Vivantis Technologies, Selangor Darul Ehsan, Malaysia), 0.3 M betaine (Sigma-Aldrich, Missouri, USA), 4 mM $MgSO_4$, 8 U *Bst* DNA polymerase (large fragment) (New England Biolabs, Massachusetts, USA), 1× ThermoPol™ Reaction Buffer (New England Biolabs), and DNA template (unless specified). The reaction mixture was incubated at 63℃ for 30 min. The LAMP products appeared as intercalating (ladder-like) bands of various base pair (bp) sizes by 2% agarose gel electrophoresis [9, 37].

2.4. Preparation of Nanogold Probes and Optimization for our LAMP-AuNP

Gold nanoparticles of 20 nm in diameter (Prime Nanotechnology, Bangkok, Thailand) were appended to the 16S-thiol probe 1, 16S-thiol probe 2, or 16S-thiol probe 3, following established methods [9, 37, 40]. In brief, all processes were performed in dark (foil covered). First, 50 µL of the 16S-thiol probe 1 (2, or 3) and 10 mL of gold nanoparticles were hybridized at 150 rpm at 50°C in a hybridization oven (Thermo Scientific, New Jersey, USA), for 22 h. Next, 1 mL of 100 mM phosphate-buffered saline (PBS), 10 µL of 10% SDS and 500 µL of 2 M NaCl, at 50°C, were added, and the mixture was hybridized at 150 rpm at 50°C for another 4 h. The mixture was centrifuged at 13,000 rpm 4°C for 30 min. The clear solution was removed, and the precipitate was washed 1-2 times in 10 mM PBS (with 100 mM NaCl and 0.01% (w/v) SDS), and centrifuged again. The pellet was finally resuspended in 10 mM PBS (100 mM NaCl and 0.01% (w/v) SDS) to give the gold nanoparticles-probe solution (AuNP) an absorbance of 0.3-0.4 at ~525 nm using a NanoDrop 2000 ultraviolet-visible (UV-vis) spectrophotometer, and was stored protected from light at 4°C.

For the colorimetric LAMP-AuNP reaction, the mixture of 5:5 (vol./vol.) ratio of LAMP:AuNP (10 µL total volume) was incubated at 63°C for 10 min, and the optimal concentration of $MgCl_2$ (40-200 mM) was then added and determined. When a sufficient concentration of Mg^{2+} was present in the absence of the LAMP product, the AuNP agglomerated and changed its color (from red to light purple or gray). However, the positive LAMP products hybridize with the AuNP via their specific complementary strands, and the agglomeration induced by the salt addition was inhibited. Positive LAMP-AuNP (this study had three different AuNP or multiplex (m)AuNP for different coliform genera: 16S-thiol probe 1, 16S-thiol probe 2, and 16S-thiol probe 3) products were identified when the LAMP-AuNP complexes were dispersed, and the mixture remained display a red color similar to when 0 mM $MgCl_2$ was added. Subsequently, the LAMP-AuNP results could be interpreted by naked eyes via their color: red (positive) or light purple/gray (negative). The UV-vis spectrophotometer was utilized to confirm the positive and negative LAMP-mAuNP results as described above, and the absorbance readings allowed computation for linear equation quantitation for number of pathogens in log CFU [9, 37].

2.5. Incorporation of PMA Assay with Crude DNA Lysis Process for Viability LAMP

Live cultures of individual (or mixed) bacterial strains were used to prepare live and dead cells at 10-fold serial dilutions from 10^0 to 10^7 CFU/mL. To obtain dead bacterial cells, the cell cultures were then heated at 95°C for 30 min in a water bath [41]. For the PMA assay, PMA solution (Biotium, California, USA) was added to 200 µL of (live or dead) bacterial culture to make a final concentration of 50 µM, incubated for 5 min in dark with occasionally inverse mixing, and exposed to a 600-W halogen light located ~15-20 cm distance for 15 min [42]. To prevent excessive heating by light exposure, the PMA-treated bacterial culture was on ice during the light exposure. Following, crude DNA lysis was performed by centrifugation to collect all bacterial cell pellets at 13,000 rpm for 5 min, washed twice with sterile deionized distilled water, and resuspended in Tris-EDTA (TE) buffer. Then, the resuspension was heated at 95°C 10 min, centrifuged to remove the cell debris at 5,000 rpm 5 min, and the supernatant was transferred to a clean microcentrifuge tube and used as a DNA template for LAMP. The supernatant was stored at -20°C [9, 25, 26].

2.6. Sensitivity and Specificity of Our PMA-LAMP-mAuNP

The sensitivity (LOD) was determined using 10-fold serial dilution cultures (0, and 10^0-10^7 CFU/mL) from the appropriate pure bacterial strains for each nanogold probe of the mAuNP (Table 1). For specificity, non-specific bacterial strains to the mAuNP probes, and no template control, were tested for the nanogold probe specificity; and dead and mixed live-dead bacterial strains were tested for the proper live vs. dead genetic discrimination in the PMA-LAMP-mAuNP assay. All PMA-LAMP-mAuNP assays were carried out at optimal conditions, as aforementioned.

2.7. ISO 16140-4 Method Validations on Ice Samples

Our PMA-LAMP-mAuNP methods for live total bacteria and *Escherichia coli* (coliform) detections were tested for an alternative method validation following the ISO 16140-4 protocol for method validation in a single laboratory, which requires testing against the reference FDA's BAM protocol [32, 33]. The ISO 16140-4 evaluated on the test sensitivity (SE_{alt}, sensitivity for the alternative method; and SE_{ref}, sensitivity for the reference method), relative trueness (RT), false positive ratio (FPR), relative level of detection (RLOD), and accuracy profile. The formulas for SE, RT, FPR and RLOD were below (provided that PA is positive agreement test number; NA, negative agreement test number; ND, negative deviation test number; PD, positive deviation test number; NA, negative agreement test number; and FP, false positive test number), and the RLOD was performed at 1, 10 and 1000 CFU of *E. coli* ATCC25922. The inclusivity test for *E. coli* included *E. coli* ATCC25922, PMK020157, and PMK020257 (the latter two strains were from Phramongkutklao Hospital).

$$SE_{alt} = ((PA+PD)/(PA+ND+PD)) \times 100 \quad (1)$$

$$SE_{ref} = ((PA+ND)/(PA+ND+PD)) \times 100 \quad (2)$$

$$RT = (PA+NA)/N \times 100 \quad (3)$$

$$FPR = (FP/NA) \times 100 \quad (4)$$

$$RLOD = LOD_{alt}/LOD_{ref} \quad (5)$$

The PMA-LAMP-mAuNP protocols were performed as aforementioned. For the reference FDA's BAM protocol for live total bacteria and *E. coli* (coliform) detection, the samples were parallel sent to the ISO certified scientific testing services at the Food Research and Testing Laboratory (FTRL), Chulalongkorn University. Noted that at the FTRL, live total bacterial detection was performed by spreading plate technique on plate count agar (PCA), incubated at 35°C 48 h, and colony forming unit (CFU) was determined based on the observed colonies per plate. Then, for coliform detection, the colonies were transferred to Lauryl sulfate tryptose broth with three serial dilutions in Durham tubes, incubated at 35°C 48 h. A presence of gas in a Durham tube suggested a coliform, and was further cultured in Brilliant green lactose bile broth at 35°C 48 h for confirmation (the cultured tube that has a gas inside represents the positive coliform presence).

3. Results and Discussion

3.1. Principle of our PMA-LAMP-mAuNP Assay for Rapid Colorimetric Live Coliform Bacteria Detection

Our PMA-LAMP-mAuNP assay integrates three molecular principles to enable rapid, visual detection of viable coliform bacteria (Figure 1). First, the PMA pretreatment step selectively differentiates live from dead cells. PMA cannot penetrate intact (viable) cell membranes, so only DNA from viable cells remains amplifiable, while the DNA of dead cells is covalently cross-linked and rendered undetectable. This step is incorporated in the crude DNA lysis thereby results in the viable bacterial cell genetic extraction, for the latter genetic amplification by PCR/qPCR and LAMP [21-26, 42]. Third, we utilized a simple and portable genetic amplification LAMP [12-14] using the specially designed primers that target universal bacteria 16S rRNA gene [29-31]. The LAMP products then hybridize with complementary 16S-thiol-oligonucleotide probes attached to nanogold particles (mAuNP). These probes are specific to different coliform genera -*Escherichia/Shigella*, *Klebsiella*, and *Enterobacter/Salmonella* (Table 2)- allowing multiplex detection within a single reaction. In the presence of target DNA, hybridization prevents nanoparticle aggregation upon $MgCl_2$ addition, resulting in a red color (positive); and in the absence of target DNA, unbound mAuNP aggregate and shift to light purple or gray (negative) [9, 37, 40]. Noted that in Figure 1, (b) the dead coliform bacteria genome were covalently bound with the PMA and could not be genetic amplified by LAMP; and (c) the live non-coliform bacteria contained no complementary coliform bases for the mAuNP and thus no LAMP:mAuNP hybridization. When there was neither genetic amplification nor specific coliform sequence, the unbounding mAuNP could be aggregated by salt ($MgCl_2$) addition and color changes. Furthermore, not only a single probe AuNP for one target of coliform genus, but also our assay utilizes multiplex probes mAuNP for targets of all coliform bacteria genera. The complementary LAMP-mAuNP hybridization analysis ensures true positive detection and hence the specificity of the assay. Moreover, quantitative estimation of viable coliform load can also be achieved by measuring absorbance at ~525 nm using UV–Vis spectrophotometry [9]. Because the assay requires only a simple halogen lamp and a portable heat source, it is well suited for local and point-of-care testings.

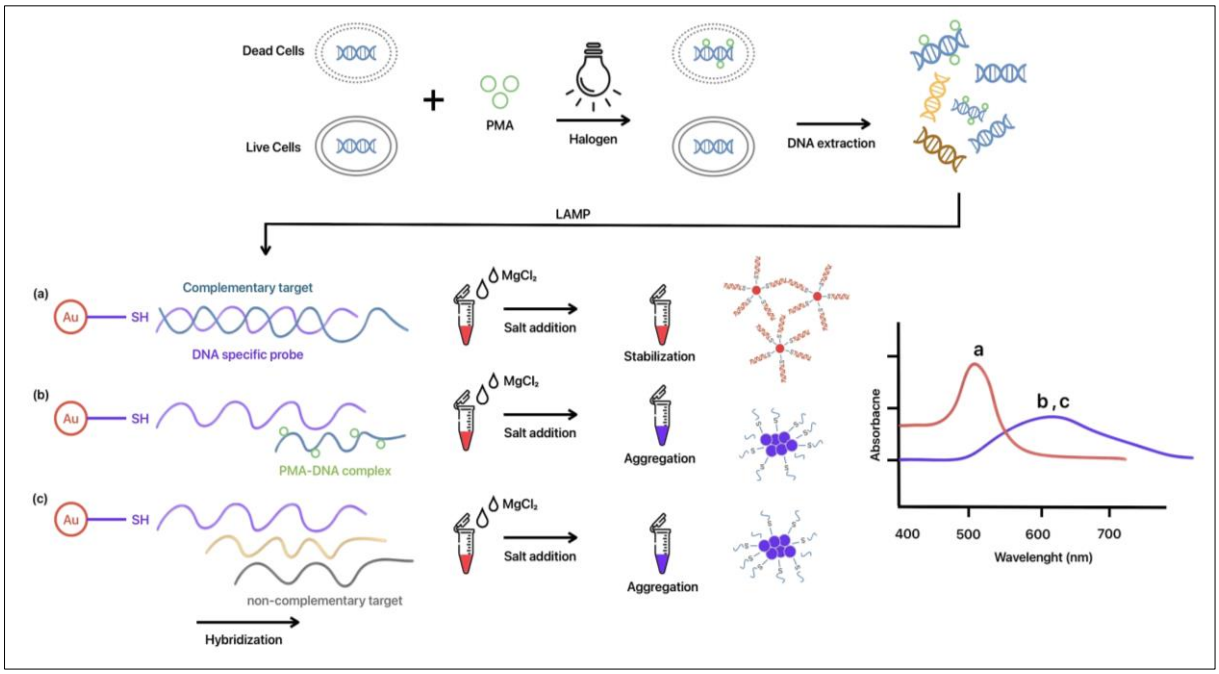

Figure 1. Schematic illustration of our PMA-LAMP-mAuNP assay, comprising PMA incorporated crude DNA lysis (viable genetic extraction, top illustrate) with universal bacteria LAMP and coliform mAuNP hybridization (genetic amplification-detection, bottom illustrate). In bottom illustrate, (a) represents a positive visual color result from live specific bacteria; (b), a negative visual color result from dead specific bacteria; and (c), a negative visual color result from live non-specific bacteria. The mAuNP represent the gold nanoparticles appended the 16S-thiol probe 1 (AuNP probe 1 is specific to genera *Escherichia* and *Shigella*), 16S-thiol probe 2 (AuNP probe 2, *Klebsiella*), and 16S-thiol probe 3 (AuNP probe 3, *Enterobacter* and *Salmonella*), respectively (details in Materials and methods).

3.2. Optimization of LAMP-AuNP Conditions

Each 16S-thiol probe was successfully conjugated to 20 nm gold nanoparticles through thiol-metal interactions, confirmed by a characteristic UV–Vis absorbance peak at ~525 nm (Figure 2a), consistent with established literatures and the observed visual color red [37, 40-42]. Because these mAuNP contain the complementary oligonucleotide sequences to the coliform bacteria genera, they hybridize with the 16S rRNA gene LAMP products that were genetically amplified from the coliform bacteria DNA template. Nonetheless, this hybridization reaction, the positive vs. negative LAMP-AuNP, could not be observed by naked eyes (Figure 2b: at 0 mM $MgCl_2$), unless the appropriate salt ($MgCl_2$) concentration was added to agglomerate non-hybridized (negative) LAMP-AuNP. We thus optimized the condition for this LAMP-AuNP positive vs. negative visual color readout by varying 40-200 mM $MgCl_2$ concentrations and found that the positive vs. negative LAMP-AuNP products were highest different in relative color readouts at 80, 120 and 160 mM $MgCl_2$ concentrations, respectively (Figure 2b). This visual color readout for the optimal concentration of $MgCl_2$ was confirmed via UV-vis spectrophotometry analysis (Figure 2c). Noted that other salts, such as NaCl, KCl, NaBr, $CaCl_2$, $MnCl_2$ and $NiCl_2$, were previously studied and could neither agglomerate the AuNP nor distinguish the positive vs. negative hybridized AuNP colors [43-45]. In this study, we also tested the other type of salt ($MgSO_4$) and found that the $MgSO_4$ could also agglomerate the AuNP and provide distinguish color readouts between the positive and negative LAMP:AuNP; however, we selected the $MgCl_2$ because it yielded the more vivid color readouts (data not shown). Moreover, this visual color genetic detection technique was reported very sensitive even as low as ~1-10 copies of the original DNA template, equivalent to PCR-GE technique [9, 37, 40, 46].

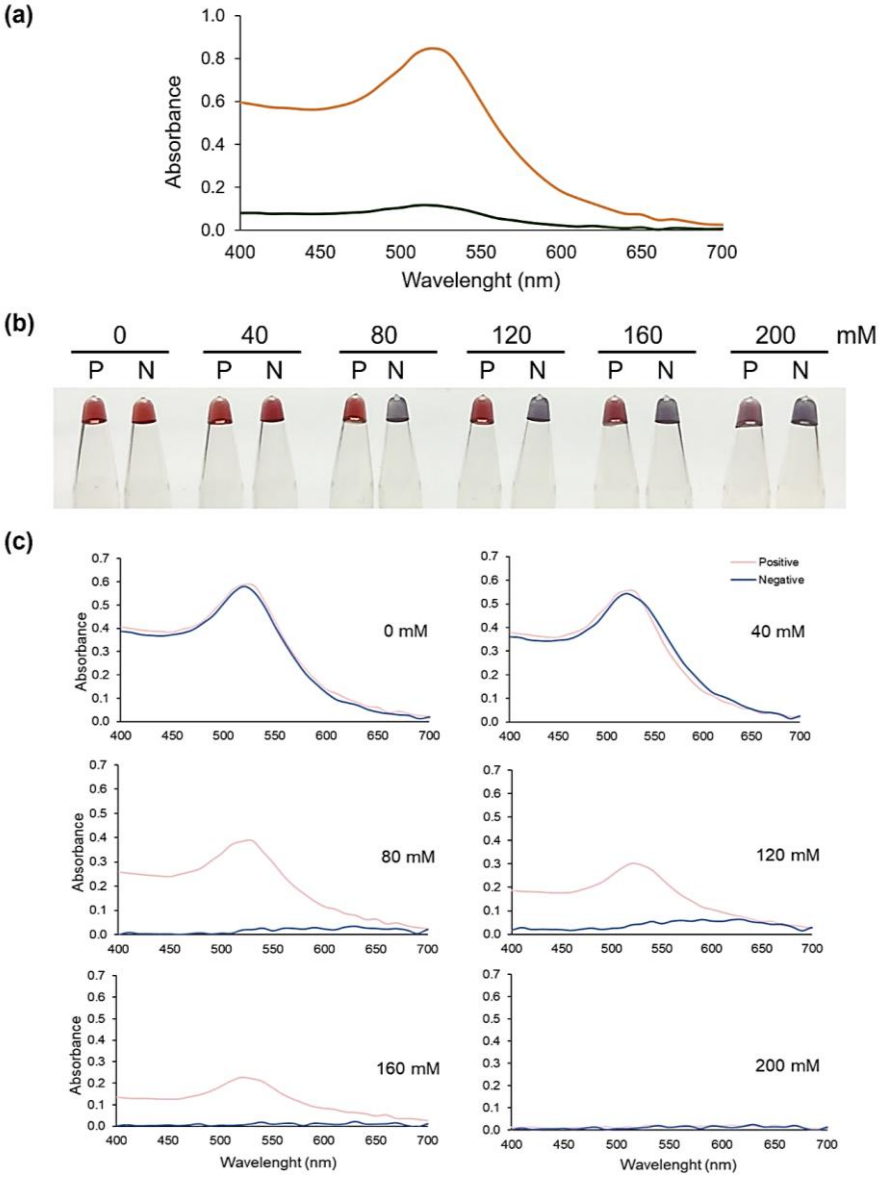

Figure 2. AuNP and determination of appropriate $MgCl_2$ concentration for positive and negative LAMP-AuNP result color readout. (a) UV-vis spectrophotometry reading of gold nanoparticles (green line) and gold nanoparticles appended the 16S-thiol probes (orange line: AuNP); and (b) visual color and (c) UV-vis spectrophotometry readings of positive and negative LAMP-AuNP products after different $MgCl_2$ concentration additions. In (b), P abbreviates positive LAMP-AuNP (containing specific DNA template), and N, negative LAMP-AuNP (containing sterile water in replace of DNA template).

3.3. Validation of our Viable-Cell DNA Extraction for Viability LAMP

We next evaluated our PMA assay with crude DNA lysis process as the viable cell genetic extraction step to allow discrimination of live vs. dead genetic (viability) LAMP. Live and dead cells of different bacterial strains from approximately 10^7 to as low as 10^0 (or 1) CFU were +PMA (or -PMA), and crude DNA lysis, followed by universal bacteria LAMP. With +PMA genetic extraction step followed by universal bacteria LAMP (PMA-LAMP), only live but dead cells could be genetically amplified by LAMP, as PMA penetrated dead bacterial cell membranes and covalently bound to the dead DNA causing the inhibition of LAMP genetic amplification (Figures 3 and 4: Live/+PMA, Dead/+PMA). In contrast, without PMA (-PMA) genetic extraction followed by universal bacteria LAMP, both live and dead cells could be genetically amplified (Figures 3 and 4: Live/+PMA, Dead/+PMA). We also found that our viable cell genetic extraction step was sensitive to as low as 1 CFU. Furthermore, because the universal LAMP could amplify any bacterial strains, we included both coliform (Figure 3) and non-coliform (Figure 4) bacteria in our viable genetic extraction method validations for the viability LAMP. Our viability LAMP (PMA-LAMP), when combined with our coliform mAuNP, then could allow the color live-coliform genetic amplification-detection (Figure 1). Consistent with previous literatures [19, 42, 47], our developed PMA-LAMP is sensitive, and more rapid and simpler than standard molecular diagnostics using genetic RNA extraction and PCR/qPCR [13, 15, 21].

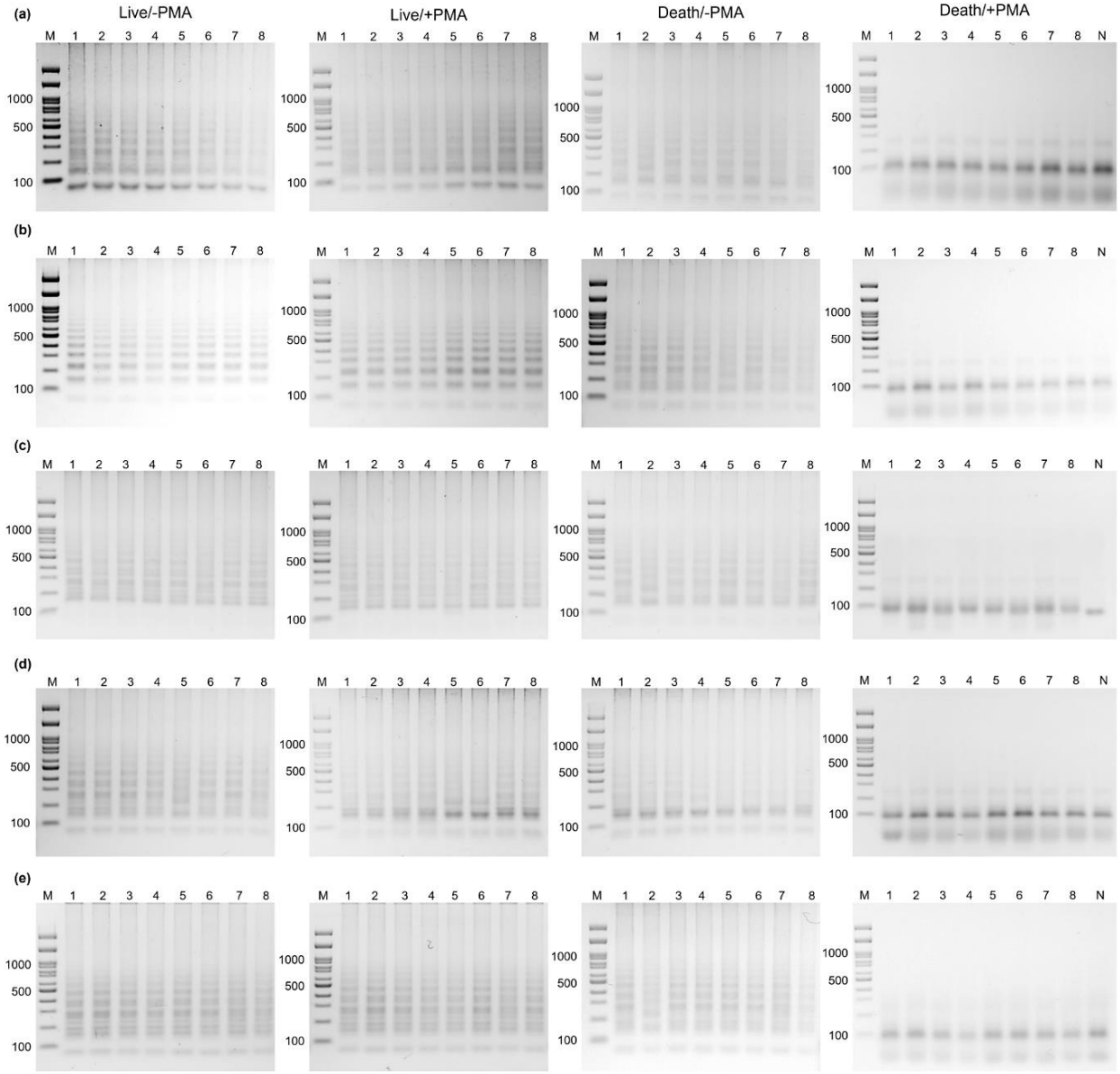

Figure 3. Detection of live and death (a) *Escherichia coli*, (b) *Shigella* sp., (c) *Klebsiella pneumoniae*, (d) *Enterobacter aerogenes* and (e) *Salmonella typhimurium* cells using PMA (+PMA) and no PMA (-PMA) and crude DNA lysis followed by LAMP.

LAMP products were analyzed by 2% agarose gel electrophoresis: lane M represented 100 bp DNA ladder marker; N, negative control (sterile water in replace of DNA template); and lanes 1-8, 10-fold serial bacteria cell dilutions from 10^7 to 10^0 CFUs. No ladder-like bands of various bp sizes for dead bacterial cells in +PMA and crude DNA lysis followed by LAMP (PMA-LAMP).

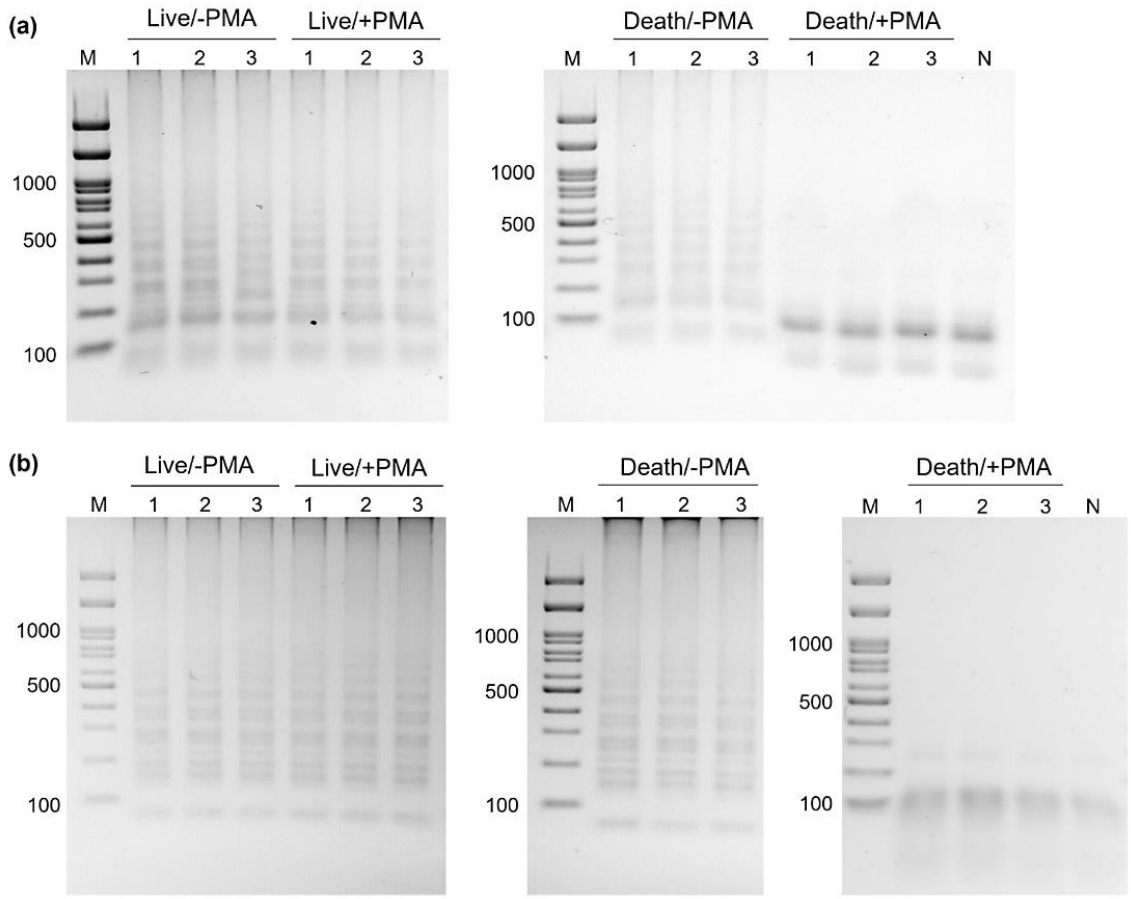

Figure 4. Detection of live and death a) *Vibrio parahaemolyticus* and (b) *Staphylococcus aureus* cells using PMA (+PMA) and no PMA (-PMA) and crude DNA lysis followed by LAMP

LAMP products were analyzed by 2% agarose gel electrophoresis: lane M represented 100 bp DNA ladder marker; N, negative control (sterile water in replace of DNA template); and lanes 1-3, bacterial cells at 10^6, 10^3 and 10^0 CFUs, respectively. No ladder-like bands of various bp sizes for dead bacterial cells in +PMA and crude DNA lysis followed by LAMP (PMA-LAMP).

3.4. Sensitivity, Specificity, and Colorimetric Quantitation of Live Coliform by PMA-LAMP-mAuNP

The final platform utilized multiplex nanogold probes to improve upon single-probe LAMP-AuNP to enable the simultaneous yet specific identifications of multiple species covering all coliform bacteria genera within a single reaction via visual colorimetric analysis. Here, we found that the sensitivity and LOD of the colorimetric live coliform bacteria PMA-LAMP-mAuNP as determined by naked eyes were proper in analyzing live and dead cells (Figures 5 to 7: +PMA showed positive color for live and negative color for dead cells, whereas -PMA showed positive color for both live and dead cells), and could analyze the presence of live bacterial cells in the starting sample from 10^7 to as low as 10^0 CFU. These visual color analyses were also validated via UV-vis spectrophotometry readings (Figures 5 to 7: absorbance peaks of tubes 1-8 at ~525 nm). This inferred that the LOD of our PMA-LAMP-mAuNP (for AuNP probes 1, 2 and 3) were all sensitive to as low as 1 CFU (equivalent to 1 coliform count). This LOD was comparable with the controlled number of coliform in drinking and food guidelines, and the LOD of the coliform bacteria culture methods [1, 5, 6]. Noted that with no PMA (-PMA), the LAMP-mAuNP showed positive color for all 10^7 to 10^0 CFU, because no PMA to intercalate the DNA template and inhibit the LAMP genetic amplification for mAuNP visual color detection.

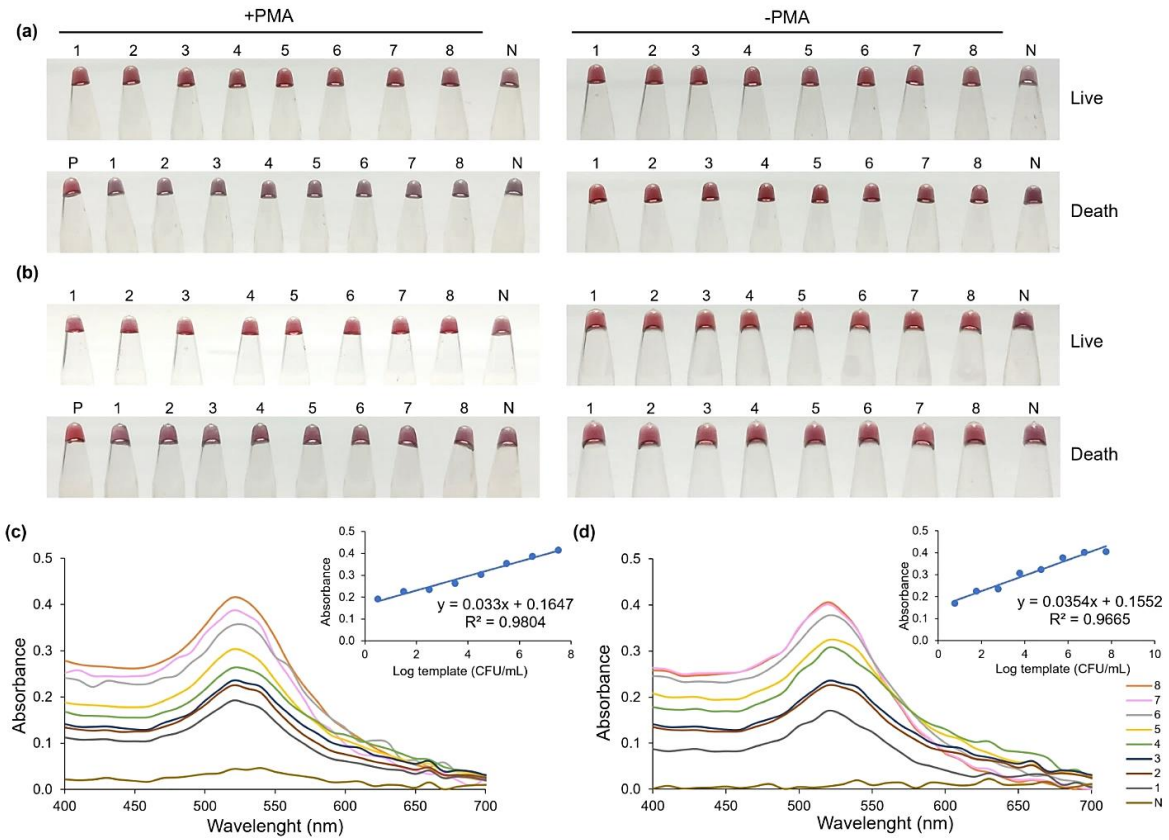

Figure 5. Limit of detection of colorimetric live coliform PMA-LAMP-mAuNP (using AuNP probe 1) for (a) *E. coli* and (b) *Shigella* sp., and (c-d) their colorimetric quantitative equations. In (a) and (b), the limit of detection could be analyzed via naked eyes (red is positive, and light purple/gray is negative). In (c) and (d), the reliable linear regression equation for log bacterial CFU template (goodness-of-fit, R2, > 0.95) was derived from the UV-vis absorbance readings at ~525 nm and 10-fold serial dilutions of the starting bacterial templates in CFU unit, and the absorbance data represented the averages from three replicate experiments. P abbreviated positive control; N, negative control (sterile water in replace of DNA template); and 1-8, 10-fold serial bacteria cell dilutions from 10^7 to 10^0 CFU.

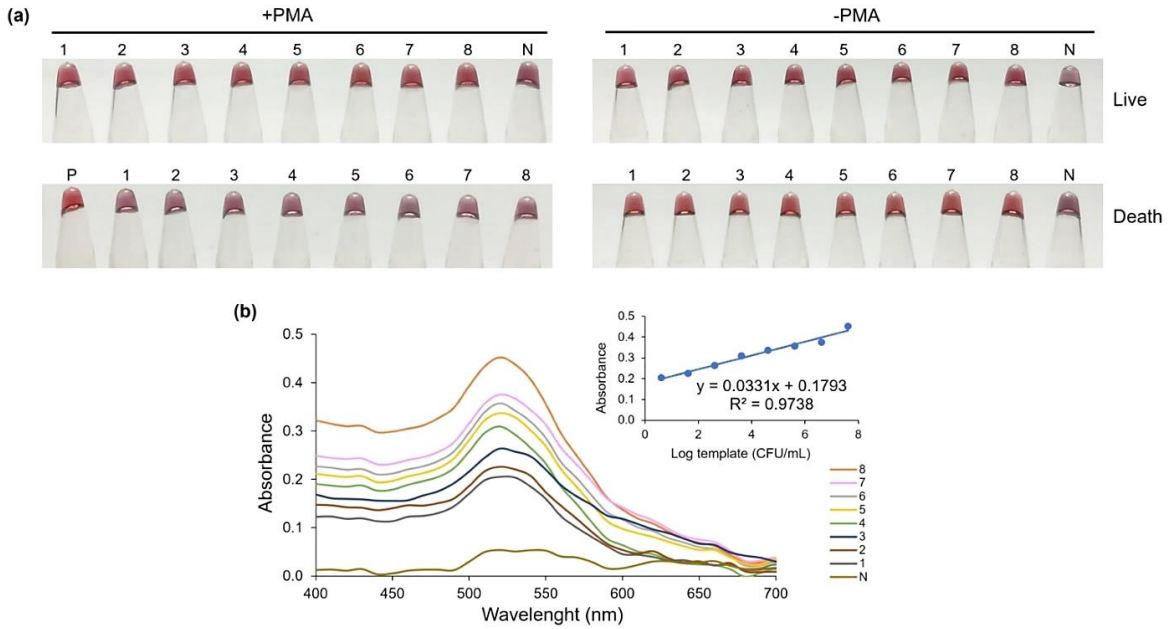

Figure 6. Limit of detection of colorimetric live coliform PMA-LAMP-mAuNP (using AuNP probe 2) for (a) *K. pneumoniae*, and (b) its colorimetric quantitative equation. In (a), the limit of detection could be analyzed via naked eyes (red is positive, and light purple/gray is negative). In (b), the reliable linear regression equation for log bacterial CFU template (R2 > 0.95) was derived from the UV-vis absorbance readings at ~525 nm and 10-fold serial dilutions of the starting bacterial templates in CFU unit, and the absorbance data represented the averages from three replicate experiments. P abbreviated positive control; N, negative control (sterile water in replace of DNA template); and 1-8, 10-fold serial bacteria cell dilutions from 10^7 to 10^0 CFU.

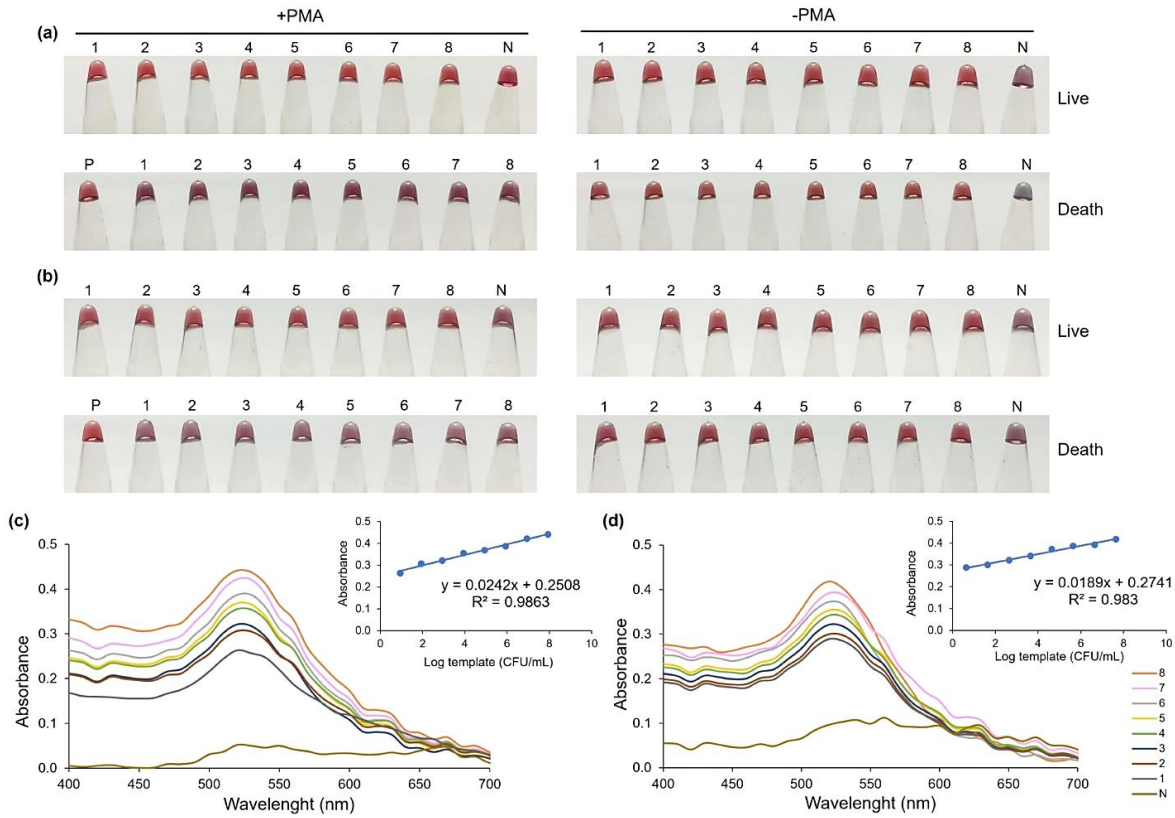

Figure 7. Limit of detection of colorimetric live coliform PMA-LAMP-mAuNP (using AuNP probe 3) for (a) *E. aerogenes* and (b) *S. typhimurium*, and (c-d) their colorimetric quantitative equations. In (a) and (b), the limit of detection could be analyzed via naked eyes (red is positive, and light purple/gray is negative). In (c) and (d), the reliable linear regression equation for log bacterial CFU template (R2 > 0.95) was derived from the UV-vis absorbance readings at ~525 nm and 10-fold serial dilutions of the starting bacterial templates in CFU unit, and the absorbance data represented the averages from three replicate experiments. P abbreviated positive control; N, negative control (sterile water in replace of DNA template); and 1-8, 10-fold serial bacteria cell dilutions from 10^7 to 10^0 CFU.

For quantitation of live coliform cell counts in CFU, the positive color for 10^7 to 10^0 CFU were analyzed for absorbance reading using UV-vis spectrophotometer and derived the linear regression equation for bacterial count quantitation from the degree of absorbance reading following established methods [9, 15, 16]. Subsequently, the linear regression equations were derived for quantitation of coliform bacteria genus *Escherichia*, *Shigella*, *Klebsiella*, *Enterobacter* and *Salmonella*, respectively (Figures 5c, 5d, 6b, 7c and 7d). The R^2 ranged from 0.9665-0.9863 and were considered reliable goodness-of-fit. The resulting consistency and the relatively parallel results across replicate experiments supported the equation derivations. Moreover, the color results of the PMA-LAMP-mAuNP were consistent with the PMA-LAMP-GE (analyses by agarose gel electrophoresis) (Figure 3a-e: Live/+PMA); however, neither the visual color by naked eyes analysis nor the ladder-band intensity by GE could alone derive the accurate linear regressive equation for coliform cell quantitation as the UV-vis spectrophotometry readings.

We acknowledged that specific testing in real sample complex matrices (milk, wastewater, and food) is essential to fully assess the robustness of the assay. The DNA extraction step employed in this study should be sufficient to recover amplifiable DNA from such matrices, enabling successful LAMP amplification and subsequent quantification through the LAMP product–AuNP interaction. Consistent with previous publications, our laboratory has experiences in applying LAMP-based quantification to real food matrices [9, 15, 16, 48, 49], and is currently extending ISO 16140-4 validation to more complex food samples using this PMA-LAMP-mAuNP platform (data not shown). Nevertheless, future works are required to warrant and conclude precisely inhibitory effects in diverse real-world food and environmental matrices, and to establish a stable quantitative linear relationship under point-of care conditions.

The specificity of our PMA-LAMP-mAuNP assays were evaluated via the specific design of each AuNP probe sequences and the BLASTN vs. non-redundant GenBank database check for specificity (Table 2), the PMA-LAMP-GE (Figure 4) and the proper identification of positive bacterial strains (Figures 5-7), as aforementioned. Additionally, other non-coliform but possible drinking-food contaminated bacteria such as *S. aureus*, *B. cereus*, *E. faecalis* and *V. parahaemolyticus*, of live and dead cells, were checked for the proper PMA-LAMP-mAuNP assay specificity, and these non-coliform bacteria species demonstrated negative color result as expected (Figure 8). Other non-coliform bacteria were also tested and demonstrated negative color result (data not shown).

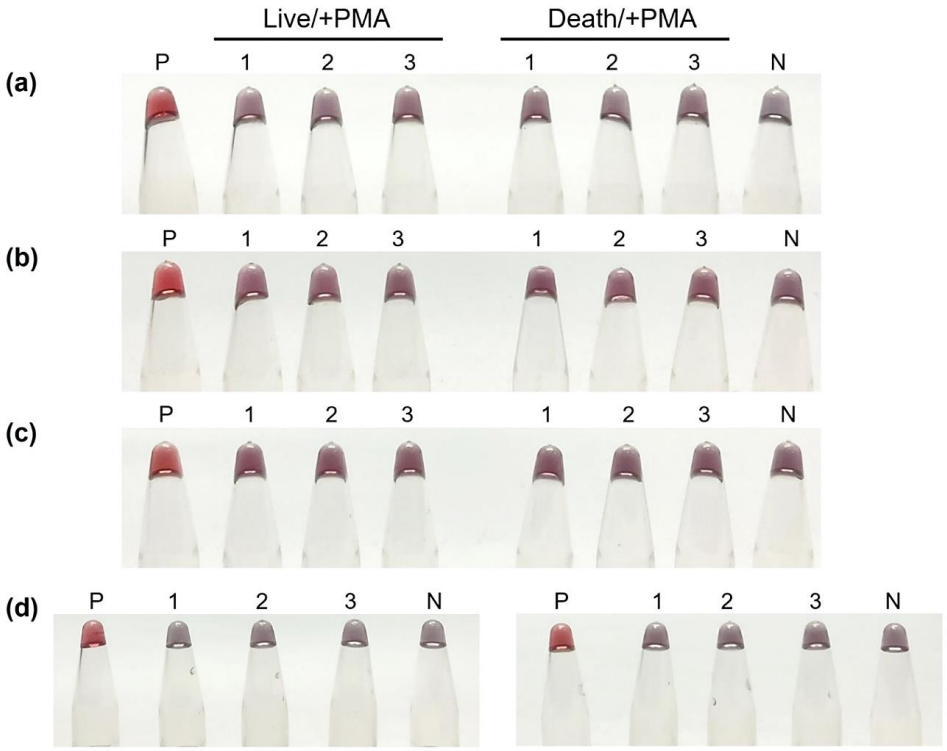

Figure 8. Specificity of colorimetric live coliform PMA-LAMP-mAuNP (using AuNP probe 1) for (a) *S. aureus*, (b) *B. cereus*, (c) *E. faecalis* and (d) *V. parahaemolyticus.* The specificity could be analyzed via naked eyes; and P abbreviated positive control; N, negative control (sterile water in replace of DNA template); and 1-3, bacterial cells at 10^6, 10^3 and 10^0 CFU, respectively.

3.5. Specificity of PMA-LAMP-mAuNP in Live or Dead, Mixed Coliform and Non-coliform Bacteria Species Detection

To simulate real-world conditions, mixed cultures containing live and dead coliform and non-coliform bacteria were examined, provided that the specific coliform genera for each AuNP were tested and the non-coliform strains represented examples of species that could contaminate drinking and food products. The dead cell cultures represented the products after heat-killed (or other bacterial eradication) processes. The objective was to confirm the absence of cross-reactivity when multiple bacterial strains were present and/or when heat treatment or bacterial eradication processes were applied to real samples. We demonstrated that the PMA-LAMP-mAuNP assay could detect coliform bacteria of the genera *Escherichia*, *Shigella*, *Klebsiella*, *Enterobacter* and *Salmonella* in all live, but not dead, cell cultures by visual color observation (Figure 9a). Further, the intensity of the positive red color measured by UV–Vis spectrophotometry was relatively consistent among live-cell experiments, reflecting the equal total number of viable coliform cells (Figure 9b). These results were in agreement with the colorimetric quantitation data (Figures 5 to 7: absorbances ~0.26-0.34 at 103 CFU). Since a single PCR-compatible assay capable of simultaneously visually detecting multiple genera for comprehensive coliform species identification is not yet available [9, 18, 23, 44, 48], and such capability is crucial for saving cost and time, we are continuing to validate the PMA-LAMP-mAuNP in local settings and point-of-care diagnostics in accordance with the International Organization for Standardization (ISO) guidelines for real-world implementation and acceptance.

3.6. ISO 16140-4 Validation of PMA-LAMP-mAuNP as Alternative Methods for Live Total Bacteria and Live Coliform Detections

The assay's performance was validated according to ISO 16140-4 standards using ice samples spiked with live *E. coli*, to validate the PMA-LAMP and PMA-LAMP-mAuNP as the alternative methods for live total bacteria and representative live coliform detections, respectively, compared against the reference FDA's BAM [32-34]. The PMA-LAMP and PMA-LAMP-mAuNP methods achieved 100% sensitivity for total bacteria and E. coli detection (Table 3: SEalt), surpassing the FDA's BAM reference method (90.91%). This high sensitivity was consistent with the ultra-sensitivity of the genetic amplification, in that 1 or a few copies of bacteria could be detected compared with the cultivation [9]. The relative trueness (RT) was found to be 95%, and the FPR was 0 (no false positive was found). With the spiked blank, low, medium, and high *E. coli* (1, 10, and 1000 CFU, respectively), the relative level of detection (RLOD), or the LODalt/LODref, was found to be lower than 1.5, which is considered acceptable (Table 3). In the

accuracy profile, the average bias values departed from the true results and the reference FDA's BAM results were found close to zero, and the calculated upper and lower beta-expectation tolerance (β-ETIalt) values of the PMA-LAMP and the PMA-LAMP-mAuNP demonstrated the data within the upper and lower reference methods' acceptable limits (ALref): total bacteria, lower β-ETIalt ranged -0.876 to -0.644 and upper β-ETIalt ranged 0.524 to 0.756 (lower ALref -0.99 and upper ALref 0.99); and *E. coli*, lower β-ETIalt ranged -0.590 to -0.489 and upper β-ETIalt ranged 0.565 to 0.666 (lower ALref -0.83 and upper ALref 0.83) (Table 3: acceptable upper and lower AL for the PMA-LAMP in total bacteria and the PMA-LAMP-mAuNP in *E. coli* detections). Moreover, the inclusivity indicated the PMA-LAMP-mAuNP methods are specific to all tested *E. coli* strains.

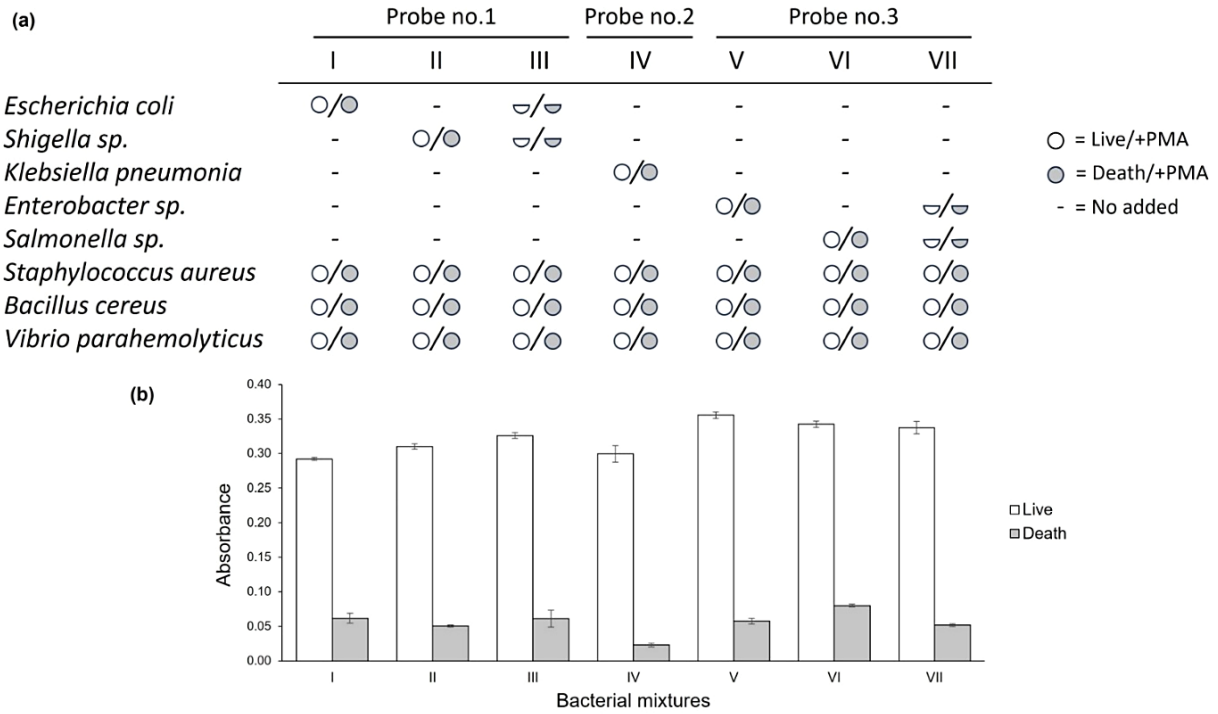

Figure 9. Specificity of colorimetric live coliform PMA-LAMP-mAuNP against live or death, mixed coliform and non-coliform bacteria species cultures. (a) Schematic diagram of live (or death) bacterial species mixture experiments and (b) the ~525 nm UV-vis absorbance reading results of the PMA-LAMP-mAuNP (data represented avg.±SD from three replicates). Each experiment contained 4-5 bacterial strain mixtures. Empty circle (or bar chart) represented live bacterial cell template; filled circle (or bar chart), dead bacterial cell template; half-circle, half of the number of cells of the full circle were added (full circle represented 10^3 CFU, half-circle 5×10^2 CFU. The latter was to make the total number of coliform cells in each experiment to be equal.

Table 3. Calculated PMA-LAMP-mAuNP test performances according to ISO 16140-4

Detections	SE_{alt} (%)	SE_{ref} (%)	RT (%)	FPR (%)	RLOD	Accuracy profile		
						Bias	Upper AL$^{\beta}$	Lower AL
Total bacteria	100	90.91	95	0	1.179*	-0.0515	Acceptable	Acceptable
E. coli	100	90.91	95	0	1.214	-0.0127	Acceptable	Acceptable

* RLOD ≤ 1.5 is considered acceptable.

$^{\beta}$ Upper (or lower) acceptable limit (AL) is considered acceptable when a beta-expectation tolerance interval of the alternative method (β-ETI_{alt}) is within this range.

Here, the FDA's BAM methods for total bacterial counts were 2 days and *E. coli* 6 days, while our alternative methods were approximately 2 hours. This simple and rapid identification might be crucial for local testings. *E. coli* was selected as the representative coliform for ISO 16140-4 validation because it is the principal indicator organism for fecal contamination in water and food safety assessments, as stipulated by the WHO and FDA. The ISO validation process requires the use of a well-characterized reference strain with established comparability to the FDA's BAM method, for which *E. coli* ATCC25922 is the standard. This provided a clear regulatory benchmark for assessing sensitivity, specificity, and method equivalence. Nonetheless, non-fecal coliforms such as *Klebsiella* and *Enterobacter* are also important contaminants in food and environmental matrices. Future studies are underway to extend ISO 16140-4 validation to these genera to further confirm the assay's robustness and general applicability across the coliform genera.

4. Conclusion

This study established and validated a rapid, low-cost, and field-deployable PMA-LAMP-mAuNP assay for the specific detection of viable coliform bacteria. The data supported the reliability of our assay (sensitivity LOD < 10 CFU and specificity 100%). The method eliminates dependence on commercial DNA or RNA extraction kits nor expensive instruments like a thermal cycler, electrophoresis apparatus, or spectrophotometer (positive and negative results could be visually informed by the naked eye). Only a halogen light bulb, a simple heat block or water bath, and readily available reagents are sufficient to perform our ultrasensitive and accurate diagnostic test at local and point-of-care settings in 80 minutes (40 min viable genetic extraction and 40 min genetic amplification-detection) while requiring minimal expertise. Indeed, we utilized a custom-designed, compact, and portable housing unit for the PMA light exposure step and the simple electric heat box for the temperature control step to support the point-of-care readiness. Still, verification of the entire platform at the point of care has remained to be operated. The products are analyzed through direct visual readout by eyes. Quantitative colorimetric analysis further enables estimation of viable coliform counts consistent with culture-based results. Validation under ISO 16140-4 confirmed high sensitivity, specificity, acceptable precision, and accuracy comparable to the FDA's BAM method, yet in a fraction of the time. Given that coliform monitoring is essential for ensuring the microbiological safety of drinking water and food products, this technology offers a practical, scalable alternative for routine surveillance and process control. As the traditional culture methods take 5-7 days, our assay presents an interesting alternative. Therefore, the developed PMA-LAMP-mAuNP platform represents a significant advancement in local and point-of-care microbiological testing, combining molecular precision with portability and affordability, facilitating real-time public health protection and industrial quality assurance.

5. Declarations

5.1. Author Contributions

Conceptualization, N.K., K.W., S.T., W.A., D.D., and S.K.; methodology, N.T., H.B. and N.S.; resources, I.C.; data curation, N.T., H.B., I.C., D.D., and N.S.; writing—original draft preparation, N.T., H.B. and N.S.; writing—review and editing, N.T., H.B., I.C., N.K., K.W., S.T., W.A., D.D., S.K., and N.S.; supervision, D.D. All authors have read and agreed to the published version of the manuscript.

5.2. Data Availability Statement

The data presented in this study are available on request from the corresponding author.

5.3. Funding and Acknowledgments

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5.4. Institutional Review Board Statement

Not applicable.

5.5. Informed Consent Statement

Not applicable.

5.6. Declaration of Competing Interest

The authors declare that there are no conflicts of interest concerning the publication of this manuscript. Furthermore, all ethical considerations, including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancies have been completely observed by the authors.

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