

METHODS MANUSCRIPT

Development of a fixation-free fluorescence *in situ* hybridization for the detection of *Salmonella* species

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Abstract

Salmonella is one of the most important infectious bacteria causing severe gastroenteritis and deaths in humans and animals, and the prompt diagnosis is crucial for effective control and treatment. The detection of *Salmonella* still depends principally on culture-based methods, which is time-consuming and laborious. Recently, polyhexamethylene biguanide (PHMB) was discovered to have cellular delivery properties and its combination with the fluorescence *in situ* hybridization (FISH) method was exploited for oligomer delivery and the rapid detection of *Salmonella* spp in this study. Cell-associated fluorescence was quantified using Volocity[®] 3-D image analysis software (Volocity 6.3, PerkinElmer, Inc.). PHMB complexed with fluorophore—labelled species-specific oligomers permeabilized freshly grown viable strains of *Salmonella* cells and mediated strong cell-associated fluorescence signals. This strategy further enabled a fixation-free protocol and hybridization in a single reaction. Using the modified FISH method, monoculture *Salmonella* strains were validated as well as detected in artificially contaminated water and milk within a turnaround period of 5 h. The method was observed to be comparable with the standard FISH technique (sFISH; $P > 0.05$). The findings suggest that fixation-free delivery and hybridization of oligomers using PHMB can provide a simplified and prompt strategy for *Salmonella* detection at the species level, and promote early management responses to the disease and appropriate antimicrobial therapy.

Keywords: *Salmonella*; polyhexamethylene biguanide; fluorescence *in situ* hybridization; bacterial diagnostics

Introduction

Salmonella is near the top of the list of pathogens of public health concern among international food standards agencies, food retailers and consumers [1]. Culture-based methods are the gold standard for isolation and identification of the pathogen [2, 3]. However, the culture of *Salmonella* usually requires pre- and selective enrichment, selective agar plating and confirmatory steps involving a series of biochemical and serological tests, which takes about 4–6 days. To overcome the lengthy analytical processing time associated with this method, newer and

rapid techniques are being developed, while older ones are modified to improve efficiency. Rapid methods used in bacterial diagnostics involve either immunological or genotypic-based techniques (polymerase chain reaction [PCR]) but none of which except fluorescence *in situ* hybridization (FISH) allows for direct *in situ* evaluation of biological samples [4, 5].

FISH using DNA oligomers has been applied as a tool in ecologic, diagnostics and environmental research [4, 6]. FISH was developed to address some of the shortcomings of culture and PCR methods [7], which is to detect nucleic acid sequences within intact cells using fluorescently labelled oligomers

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complementary to RNA target sequences [4]. For bacterial applications, ribosomal RNA is used as targets for FISH oligomers due to its well-known primary and secondary structures and high copy number [8–10]. Nonetheless, due to the stringent and complex nature of bacterial cell barriers, DNA oligomer delivery remains a significant challenge for the direct detection of genetic markers in microorganisms by FISH [11]. Cell fixation with paraformaldehyde (PFA), ethanol, methanol or detergents was introduced to enable oligomer penetration of cell barriers but remains unclear to what extent they affect bacterial integrity [12]. Other protocols used to improve oligomer permeabilization and uptake include exposure to enzymes, heat or non-detergent chemical. Certain bacterial species require even harsher cell pre-treatments to hydrolyze the thicker peptidoglycan layer before fixation and delivery [13]. To improve upon these deficiencies peptide nucleic acid (PNA), a DNA mimic with favourable stability and hybridization properties was developed [14], and applied in combination with the standard FISH (PNA-FISH) protocols. PNA oligomers can perform better than DNA in several applications, possibly due to a better cell or target rRNA accessibility or stability. Nevertheless, due to the time-consuming pre-enrichment, permeabilization and fixation steps typically required before identification [15, 16], there is a need to further improve the FISH technique. In particular, it would be desirable to reduce contamination or inhibition and promote direct intracellular oligomer delivery unrestricted by variable permeabilization and fixation protocols.

In this study, we modified the standard FISH method by using polyhexamethylene biguanide (PHMB). Figure 1 describes the structure of PHMB. It is a membrane-active cationic polymer used as a preservative and biocide, but is also an important drug used in several topical applications [17]. PHMB has been described to form nanoparticles with a broad range of molecules including nucleic acids and promote cellular internalization [18]. Recently, the novel property of PHMB as a macromolecule delivery agent at low milligram per millilitre concentrations has been reported [19]. PHMB can form noncovalent interactions with macromolecules through extensive hydrogen bonding, electrostatic and hydrophobic bonding potential [19]. Considering these unique properties, we hypothesized that PHMB would interact and complex with DNA oligomers, facilitate delivery into metabolically active bacteria and detection through a simplified, fixation-free FISH procedure in a single reaction.

Materials and methods

Bacterial strains and culture maintenance

The details of the bacterial strains used in this study are summarized (Table 1). The strains were maintained in 30% glycerol

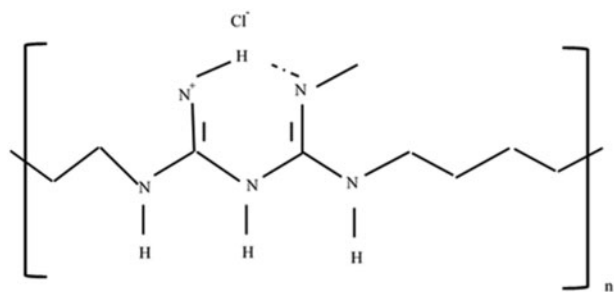


Figure 1: PHMB is a cationic polymer of repeating hexamethylene biguanide groups ($n = 13.8$; (C₈H₁₇N₅)_n). Molecular weight = 3035 g/mol. The alternative chemical name is polyhexanide.

at -80°C and grown on Luria-Bertani (LB; Sigma–Aldrich, UK) agar plates at 37°C for 16–18 h. Plates were stored at 4°C and fresh culture plates from stocks prepared every 2 weeks for experiments. Before delivery and FISH studies, bacteria species identities were validated by PCR.

FISH oligomers and PHMB

FISH oligomers targeting the 23S RNA were selected from literature and evaluated against all currently available sequences of *Salmonella enterica* using the National Centre for Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov/BLAST/>). Oligomers with the highest predicted specificity to query gene were selected (sal 2 and salm 63). To ensure the selected oligomers were best for this study, the theoretical specificity and sensitivity were assessed by conducting *in silico* alignments against sequences present in the large subunit 23S/28S databases (SILVIA) using the probeCheck software program as demonstrated by previous authors [3, 5, 20–22]. A computational analysis of selected FISH oligomers was necessary and performed to predict the outlook of the *in vitro* experiments, and the degree of sequence complementarity to target organism—i.e. the highest number of *Salmonella* sequences the probe can detect (sensitivity) as well as non-target *Salmonella* sequences detected (specificity) [23]. Scrambled mismatched sequences (negative control oligomers) were designed and evaluated *in silico* for non-specificity to the target RNA. Both positive and negative control oligomers were mono labelled at the 5'-end with carboxyfluorescein (6'-FAM) and synthesized by Sigma–Aldrich, UK. The details of the FISH oligomers used in this study are described in Table 2. PHMB and PHMB-FITC (FITC-Fluorescein isothiocyanate) were acquired from Tecrea Ltd, UK.

FISH oligomer delivery assay

Overnight bacterial cultures of *Salmonella* and *Escherichia coli* strains were diluted in Muller Hinton Broth (1:100; MHB, Sigma–Aldrich, UK) and incubated at 37°C for 2 h. Aliquots of the cultures were standardized at $\text{OD } 600\text{-nm} \sim 0.2 \pm 0.050$, washed twice in phosphate buffer saline (PBS) solution ($\text{pH} = 7.4$; PBS, Sigma–Aldrich, UK) by centrifugation at $13000 \times g$ for 5 min. Meanwhile, PHMB-oligomer at $2.8 \mu\text{g/ml}:0.4 \mu\text{M}$, $6 \mu\text{g/ml}:1 \mu\text{M}$ and $7 \mu\text{g/ml}:1 \mu\text{M}$ were prepared and complexed in sodium PBS, $\text{pH } 12$ (Sigma–Aldrich, UK) at 37°C for 30 min. The complexed mixtures were added to the cells and further incubated at 37°C in a dark chamber with continuous shaking at $250 \times g$ for 2 h.

Cell viability tests

Temperature dependence assay

Aliquots of standardized *Salmonella* cells ($\text{OD } 600\text{-nm} \sim 0.2 \pm 0.050$) were washed twice in PBS solution ($\text{pH} = 7.4$) by centrifugation at $13000 \times g$ for 5 min, and later treated with PHMB:oligomer complex at concentration $2.8 \mu\text{g/ml}:0.4 \mu\text{M}$. Tubes containing the mixtures were incubated at varying temperatures conditions of 4°C (on ice), 25°C and 37°C with continuous shaking at $250 \times g$ for 2 h. The cells were visualized by microscopy and cell-associated fluorescence quantified using Volocity[®] 3-D image analysis software (Volocity 6.3, PerkinElmer, Inc.).

Sodium azide treatment

Aliquots ($100 \mu\text{l}$) of standardized *Salmonella* cells ($\text{OD } 600\text{-nm} \sim 0.2 \pm 0.050$) were treated with different concentrations of

Table 1: Details of bacterial strains used for oligomer delivery and FISH studies

Strains	Genotypic characteristics	Source	Purpose
<i>Salmonella enterica</i> Typhimurium NK262C	n/a	SGSC 1539 ^a	Delivery and FISH assays
<i>Salmonella enterica</i> Typhimurium 7953S	Virulent phoPQ; Tn10 mutant of serovar Typhimurium LT2		
<i>Salmonella enterica</i> Typhimurium TT 103729	n/a		
<i>Salmonella enterica</i> Typhimurium LT2	Wild type strain		
<i>Escherichia coli</i> K12	Wild type strain	SGSC 1539 ^a	

^aSGSC, *Salmonella* Genetic Stock Centre, University of Calgary, Canada.

Table 2: Oligomer sequences used for FISH studies

Oligomer name	Oligomer type	Specie target	Oligomer sequence	Target gene	Purpose	Length	Reference
Sal 3	DNA	<i>Salmonella</i>	aatcacttcacctactg	23S rRNA	Positive control	18mer	[24]
Salm 63	DNA	<i>Salmonella</i>	tcgactgacttcagctcc	23S rRNA	Positive control		[25]
Mis sal 3	DNA	None	aacgctctcactctgata	None	Negative control		This study
Mis sal 32	DNA	None	actcgtcacatctatga	None	Negative control		This study

sodium azide (NaN₃; Sigma-Aldrich, UK) from 0 to 10 mg/ml according to the past study [26]. Treated cells were incubated overnight at 37°C in a 96-well plate reader Bio-Tek Powerwave X3401 spectrophotometer with agitation and OD readings monitored every 5 min. Later, cells were washed and resuspended in 100 µl PBS. Bacteria cells were then treated with PHMB:oligomer complex and incubated for 2 h with continuous shaking as previously described. Cell-associated fluorescence was quantified. Untreated bacteria and 4% PFA (Life Technologies, UK) fixed cells treated with PHMB-oligomer complex were included as controls.

Resazurin cell viability assay

First, aliquots of *Salmonella* were treated with PHMB:oligomer complexes (at concentrations 2.8 µg/ml:0.4 µM and 7 µg/ml:1.0 µM) and observed for associated fluorescence. Later, volumes of 100 µl of the same treated cultures were deposited into a 96-well plate and 10 µl volumes of resazurin (10% of the initial volume in the well) added. The plate was incubated overnight at 37°C. Fluorescent resazurin was quantified as relative fluorescence units (RFUs) at excitation wavelength 530–570 and emission wavelength 590–620 nm using an infinite[®] M200 PRO, Tecan fluorometer. Results were analyzed using Magellan 7 software. Untreated cells grown in MHB and heat-killed cells were used as controls.

Standard FISH

Fresh bacteria were resuspended in 750 µl PBS and fixed by adding 250 µl of 4% PFA fixative. The cell suspensions were incubated for 1 h on ice and then rinsed in PBS twice by centrifugation (at 13 000 × g for 5 min). A total of 1 ml volume of 50% ethanol was added to the cells and stored at 20°C until further use [8, 23]. For hybridization, the fixed cells were resuspended in 100 µl hybridization solution containing 0.9 M sodium chloride, 20 mM Tris/HCl (pH 7.2), 35% formamide 0.01% SDS and 5.9 ng/µl oligomer and tubes incubated at 46°C for 3 h. Cells were washed in 100 µl pre-warmed standard wash solution (0.9 M sodium chloride, 20 mM Tris/HCl [pH 7.2] and 0.01% SDS) at 46°C for 30 mins and the associated cell fluorescence estimated.

Modified FISH

Bacterial cultures after PHMB-oligomer complex treatment (2.8 µg/ml:0.4 µM) at 37°C for 2 h were harvested and centrifuged at 13 000 × g for 5 min. Cells pelleted were resuspended in ×1 PBS (500 µL), then washed twice by centrifugation (at 13 000 × g for 5 min). Afterward, 100 µl of pre-warmed wash buffer solution (containing 0.9 M NaCl, 20 mM Tris/HCl pH 7.2 and 0.01% SDS) was added to the cells and incubated at 50°C for 30–45 min in a water bath. Vortexing was carried out every 15 min. Cells were later rinsed and the associated cell fluorescence estimated.

Artificially contaminated water and milk

Volumes of 9 ml sterile water were artificially contaminated with 1 ml volumes of early log *Salmonella* cells (OD 600 nm ~ 0.2 ± 0.050). Artificially contaminated water samples (100 µl) were treated with PHMB-oligomer complex (100 µl) at this time at 55°C for 30 min according to the previous study [6]. Post-hybridization wash was performed in pre-warmed wash buffer solution (consisting of 0.9 M sodium chloride, 20 mM Tris/HCl and 0.01% SDS) at 55°C for 30 min. Similarly, milk powder (1 g; Sainsbury, UK) was reconstituted in 8 ml of sterile water. Reconstituted milk was artificially contaminated with 1 ml volume of *Salmonella*. The artificially contaminated milk samples were further diluted 10-fold before PHMB-oligomer complex treatment to reduce unacceptable auto-fluorescence. Post-hybridization wash was performed in pre-warmed wash buffer solution (consisting of 0.9 M sodium chloride, 20 mM Tris/HCl and 0.01% SDS) at 55°C for 30 min in a water bath.

Microscopy and quantification of cell-associated fluorescence

Bacteria cultures treated with PHMB-oligomer complexes were counterstained by using 4',6-diamidino-2-phenylindole (DAPI, 1 µM), and aliquots (3–5 µl) deposited on microscope slides. ProLong[®] Gold Antifade Reagent (Life Technologies[®]) was applied directly to the cells to reduce photobleaching and coverslips affixed. For best resolution and low fluorescence background, a drop of LDF immersion oil was applied onto coverslips (Leica, Germany). A Leica DM4000B upright fluorescence microscope (Leica, Wetzlar, Germany) equipped with a mercury

lamp and filters were used to view cells at magnification $\times 63$. Two microscope filter sets were used; the first was used to display DAPI-stained cells at excitation wavelength 340–380 nm and emission wavelength 450–490 nm and the second was used to display FITC at excitation wavelength 460–500 nm and emission wavelength 512–542 nm. Images were captured using Zeiss AxioVision software and further processed as tagged image files format (TIFF). Quantitative analysis for cell-associated fluorescence was performed using Volocity[®] 3-D image analysis software (Volocity 6.3, PerkinElmer, Inc.). Quantitative data were generated by first counting total cells stained with DAPI, followed by cells stained with FITC using the blue and green filters, respectively, on the Volocity 6.3.3 software. The percentage of positive green fluorescent cells was calculated as a total number of green fluorescent cells (FITC) divided by total cells stained blue (DAPI) $\times 100$.

Statistical analysis

Quantitative data generated were presented as mean \pm SEM. Statistical analysis was performed by using one-way analysis of variance (1-way ANOVA) for the comparison of means for more than two independent groups with a single outcome. Two-way analysis of variance (2-way-ANOVA) was used for the comparison of means of more than two independent groups with two or more outcomes. Post hoc analysis using Dunnett's multiple comparisons test was performed. All analyses were conducted using statistical packages GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA, USA). Differences were scored statistically significant if the P-value was ≤ 0.05 .

Results

In silico determination of the specificity and sensitivity of Sal 3 and salm 63 oligomers

To determine the theoretical specificity and sensitivity of sal 3 and salm 63 oligomers used for delivery and FISH studies, *in silico* evaluation using updated databases in ProbeCheck programmes was conducted [5]. The analysis indicated both oligomers presented acceptable levels of specificity and sensitivity. There was no cross-reactivity or hybridization with the 16S rRNA sequences present in the small subunit (SSU ribosomal RNA, 739633 16S rRNA) database. Despite the 100% sequence similarity, sal 3 detected only 270 *Salmonella* sequences out of 298 hits. Salm 63 identified 267 *Salmonella* sequences out of 314 hits. Salm 63 cross-matched with four bacteria strains of different species, which were *Enterobacter*, *Cronobacter* spps, *Yersinia enterocolitica* and *Plesiomonas shigelloides*. The theoretical specificities and sensitivities of the various oligomers were determined using ProbeCheck programme, 23S/28S rRNA—SILVA, Version 111 and presented in [Supplementary Table S1](#).

Delivery of oligomers into bacteria

In this study, we assessed PHMB to facilitate the delivery of oligomers (sal 3 and salm 63 sequences modified with 6'-FAM fluorescein) into viable bacteria cells. PHMB concentrations from 0 to 7 $\mu\text{g/ml}$ were tested. PHMB-mediated oligomer delivery into early log bacteria cells (incubated for 2 h) at concentrations 6 $\mu\text{g/ml}$:1 μM and 7 $\mu\text{g/ml}$:1 μM prepared in phosphate buffer pH 12. Cells were noticed to produce strong cell-associated green fluorescence and were motile under a fluorescence microscope, which indicated delivery and to an extent some level of viability. Similarly, cells treated with PHMB-FITC (positive control)

displayed strong fluorescent signals ([Fig. 2a](#)). The delivery efficiencies at 6 and 7 $\mu\text{g/ml}$ concentrations of PHMB were estimated as follows: *Salmonella* NK262 (96% and 98%), *Salmonella* 7953S (91% and 94%), *Salmonella* TT10329 (88% and 94%), *Salmonella* LT2 (72% and 95%) and *E. coli* K12 (71% and 86%) ([Fig. 2b](#)). In contrast, cells treated with oligomers or PHMB only showed little or no fluorescence. The comparative analysis demonstrated a significant level of delivery into cells treated with PHMB-oligomer complexes and PHMB-FITC than untreated ($P < 0.001$).

To further ensure PHMB delivery was possible into metabolically active cells, PHMB at lower concentrations were tested. For this experiment, three strains, namely *Salmonella* NK262C, *Salmonella* 7953S and *Salmonella* TT 103729 were used. The bacterial growth curves in the presence of several PHMB concentrations (0.0, 1.4, 2.1, 2.8 and 7 $\mu\text{g/ml}$) were presented in [Fig. 3](#). Bacterial cultures were viable, although growth took longer than untreated cultures indicating inhibition by PHMB. Nevertheless, PHMB at concentration 2.8 $\mu\text{g/ml}$ supported both delivery and cell viability, and this concentration was used for subsequent studies.

PHMB at a lower concentration of 2.8 $\mu\text{g/ml}$ was complexed with oligomers sal 3 and salm 63 to further determine the efficacy of delivery. The delivery of sal 3 was 89% and 78% for *Salmonella* and *E. coli*, respectively. For salm 63, efficiency was estimated as 84.2% for *Salmonella* NK262C, other *Salmonella* strains 7953S and TT10329 (90%) and *E. coli* (87%). For both oligomers, the cell-associated fluorescence was significantly higher ($P < 0.001$) in all the bacterial strains treated than untreated. Also, deliveries were similar ($P > 0.296$) across all bacterial strains treated.

Cell viability and effect on PHMB delivery

To determine if cell metabolism or viability was needed for improved PHMB delivery, three assays including temperature treatment, sodium azide and resazurin cell viability assays were performed.

Salmonella cultures were treated with PHMB-oligomer complexes at 4°C, 25°C and 37°C. There was a reduction in cell-associated fluorescence as temperature decreased. Bacteria cells treated with the complex at 25°C and 4°C produced drastic reduction in delivery with little or no fluorescence unlike cells grown at 37°C, which is the optimal cell temperature for enzymatic activity ([Fig. 4a](#)). Almost an 8-fold and 4-fold decrease in delivery efficiency was observed at 4°C and room temperature, respectively, compared with at 37°C ($P < 0.0001$; [Fig. 4b](#)). These findings suggested that PHMB entry into cells could be energy-dependent and may require active metabolism for efficient intracellular delivery.

For cells treated with different concentrations of NaN_3 , the highest levels of cell-associated fluorescence signals ranged from 37.2% to 67.0%, and were observed at doses ≤ 6 mg/ml. Above this concentration, delivery became drastically reduced with little or no fluorescence (3.4–10.4%, $P < 0.001$). PFA-treated cells (control negative) showed little or no delivery or fluorescence signals, while PHMB-oligomer complex without NaN_3 presented high levels of fluorescence signals estimated at 83.3% ([Fig. 5a and b](#)). A regression analysis conducted showed a dose effect of NaN_3 on bacteria growth ([Fig. 5c](#)). Higher NaN_3 concentrations reduced bacteria growth rates and delivery further suggesting that PHMB may require cell viability delivery ([Supplementary Fig. S1](#)) presents the effect of various concentrations of NaN_3 on *Salmonella* growth curves.

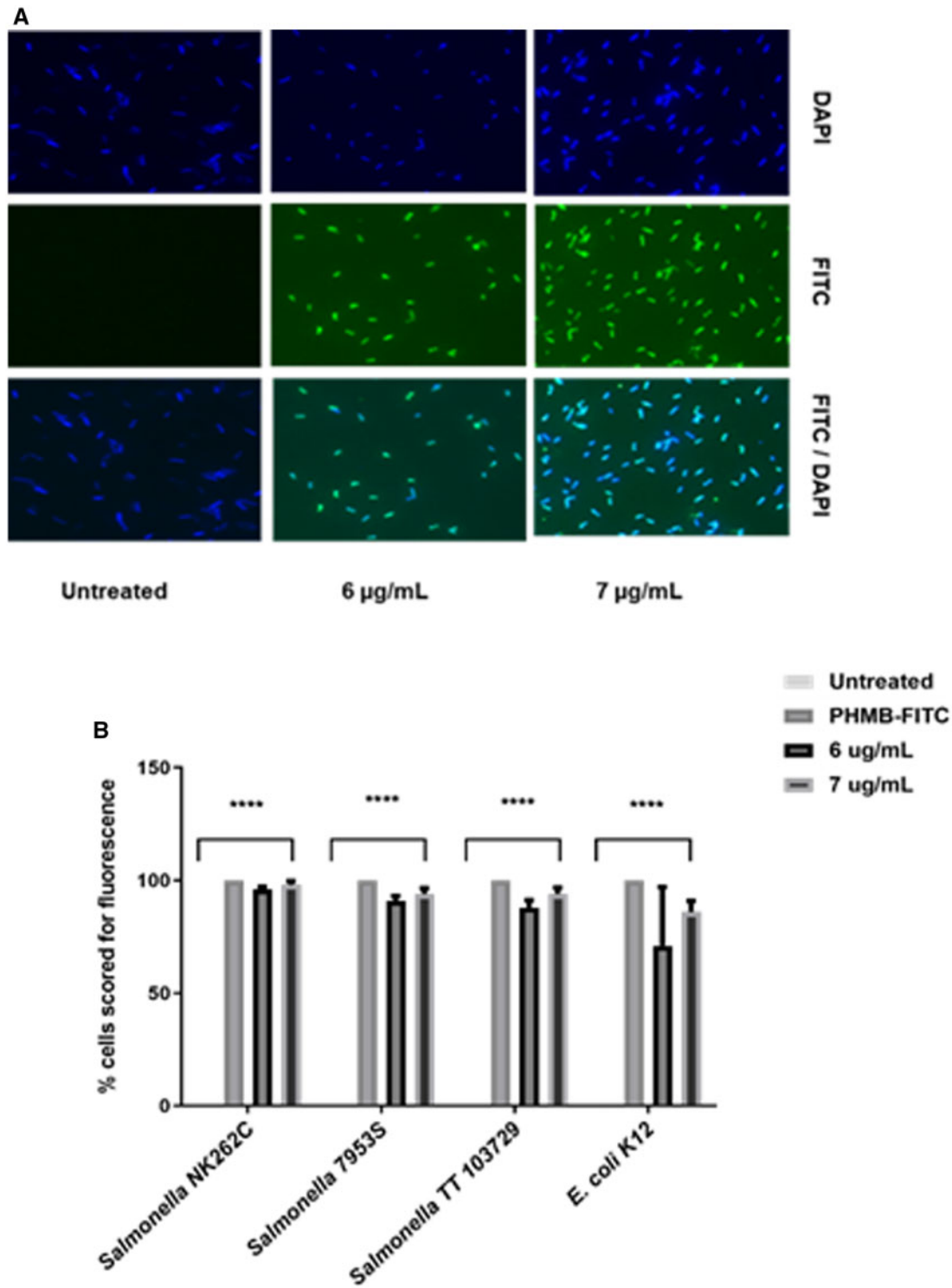


Figure 2: PHMB-mediated oligomer delivery into bacteria. (a) Fluorescence microscopy of PHMB delivery of oligomer into *Salmonella* NK262C cells at concentrations 6 and 7 $\mu\text{g/ml}$. (b) Quantitative measurements of PHMB delivery efficiency for various bacteria strains tested. Cell-associated fluorescence was quantified using velocity 6.3.3 software. PHMB-FITC and untreated cells were used as control positive and negative, respectively. Data are presented as the mean and standard error of the mean for biological replicates ($n = 3$).

Lastly, the resazurin assay performed indicated fluorescence intensity generated by cells treated with PHMB-oligomer complex at concentrations 2.8 $\mu\text{g/ml}$:0.4 μM and 7.0 $\mu\text{g/ml}$:1.0 μM reduced 5- and 10-fold in contrast with the untreated (positive

control; $P < 0.0001$; Fig. 6a). Nevertheless, cells treated at 2.8 $\mu\text{g/ml}$ showed higher level of activity than 7.0 $\mu\text{g/ml}$ ($P < 0.016$). No fluorescence signals were observed in heat-killed cells and sterile MHB (negative controls).

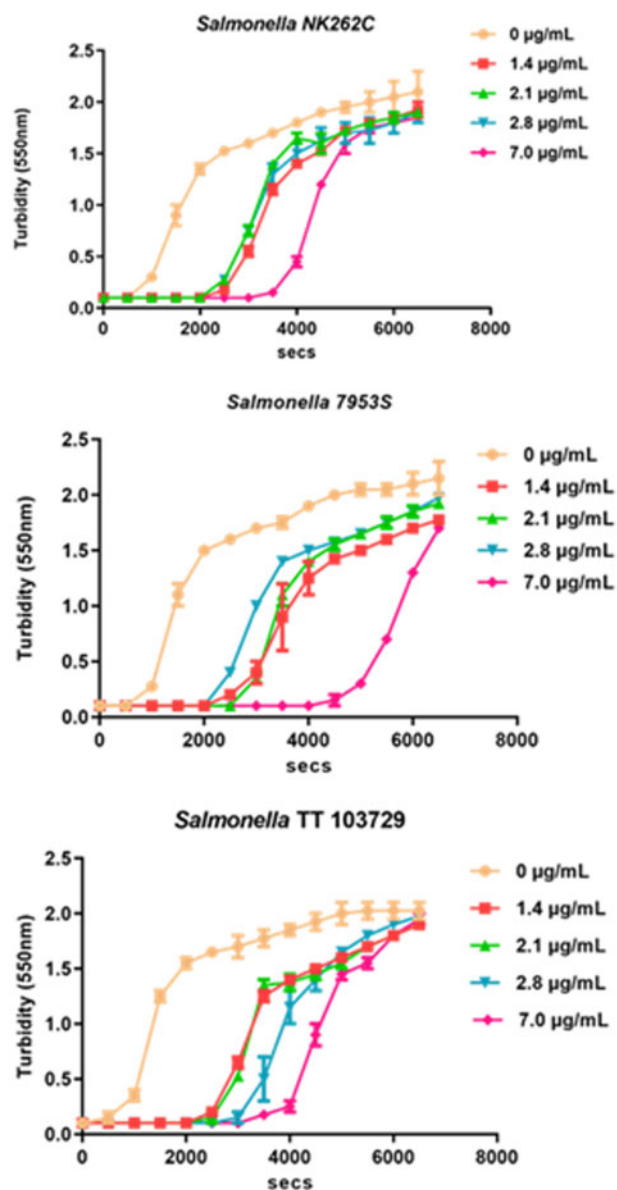


Figure 3: Effects of PHMB concentrations on bacterial growth. Growth curve analysis of various *Salmonella* strains (NK262C, 7953S and TT03729) treated with different PHMB concentrations 0, 1.4, 2.1, 2.8 and 7 µg/ml. OD readings at 550 nm were taken after overnight incubation. Bacteria cells were observed to be viable, although growth rates took longer than untreated ($P < 0.0001$). PHMB at 2.8 µg/ml supported both viability and delivery. 7.0 µg/ml was used for growth comparisons. Data are presented as the mean and standard error of the mean for biological replicates ($n = 2$).

PHMB enhanced fixation-free FISH and subsequent detection of *Salmonella*

To determine if PHMB could promote a fixation-free hybridization protocol and subsequent detection of *Salmonella*, post-hybridization wash conditions were optimized and cell-associated fluorescence quantified. Wash buffer 0.7 M NaCl, 20 mM Tris-HCl pH 7.2 and 0.01% SDS at 50°C for 30–45 min produced the strongest signal-to-noise ratio and removal of non-target mismatched oligomers. Sal 3 and salm 63 accurately detected all monocultures of *Salmonella* with strong visible cell-associated fluorescence signals (Fig 6b and c). No cross-reactivity with closely related *E. coli* K12 strain was evident

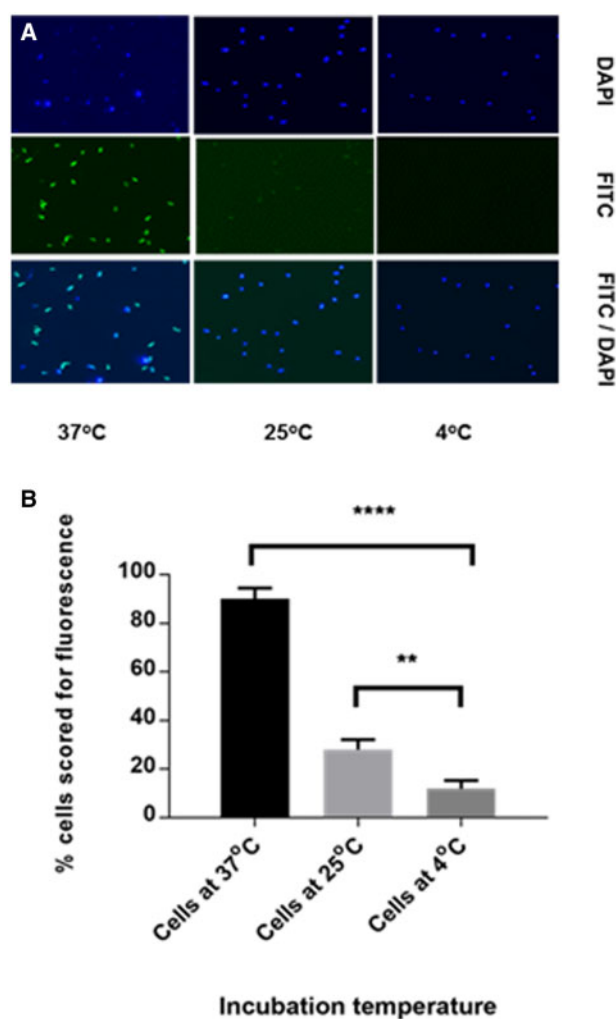


Figure 4: Effects of temperature on PHMB oligomer delivery. (a) Fluorescence microscopy of *Salmonella* NK262C cells treated with PHMB: oligomer complex at various temperatures 37°C, 25°C and 4°C. (b) The delivery efficiency of PHMB quantifications as cell-associated fluorescence was carried out using velocity 6.0 imaging software. Data are presented as the mean and standard error of the mean for biological replicates ($n = 3$).

except for salm 63 in which some level of fluorescence was observed. For the sal 3, the cell-associated fluorescence after post-hybridization wash was quantified as 87%, 91%, 82% and 13% for *Salmonella* NK262C, *Salmonella* 7953S, *Salmonella* TT103729 and *E. coli* K12, respectively. The results observed for salm 63 were 80%, 65%, 87% and 59% for *Salmonella* NK262C, *Salmonella* 7953S, *Salmonella* TT103729 and *E. coli* K12, respectively.

For mismatched oligomers, mis sal 2 and mis sal 32, drastic reduction in fluorescence signals after wash was observed for all *Salmonella* strains and non-target *E. coli* K12. However, mis sal 32 produced some level of non-specific background signals with *E. coli* K12, despite producing no hits in both the NCBI and Probecheck databases. For mis sal 2, the cell-associated fluorescence was 5%, 7%, 19% and 9%, while mis sal 2 was 10%, 7%, 24% and 37% for *Salmonella* NK262C, *Salmonella* 7953S, *Salmonella* TT103729 and *E. coli* K12, respectively. Table 3 provides a comparison of the modified FISH (mFISH) technique to other methods.

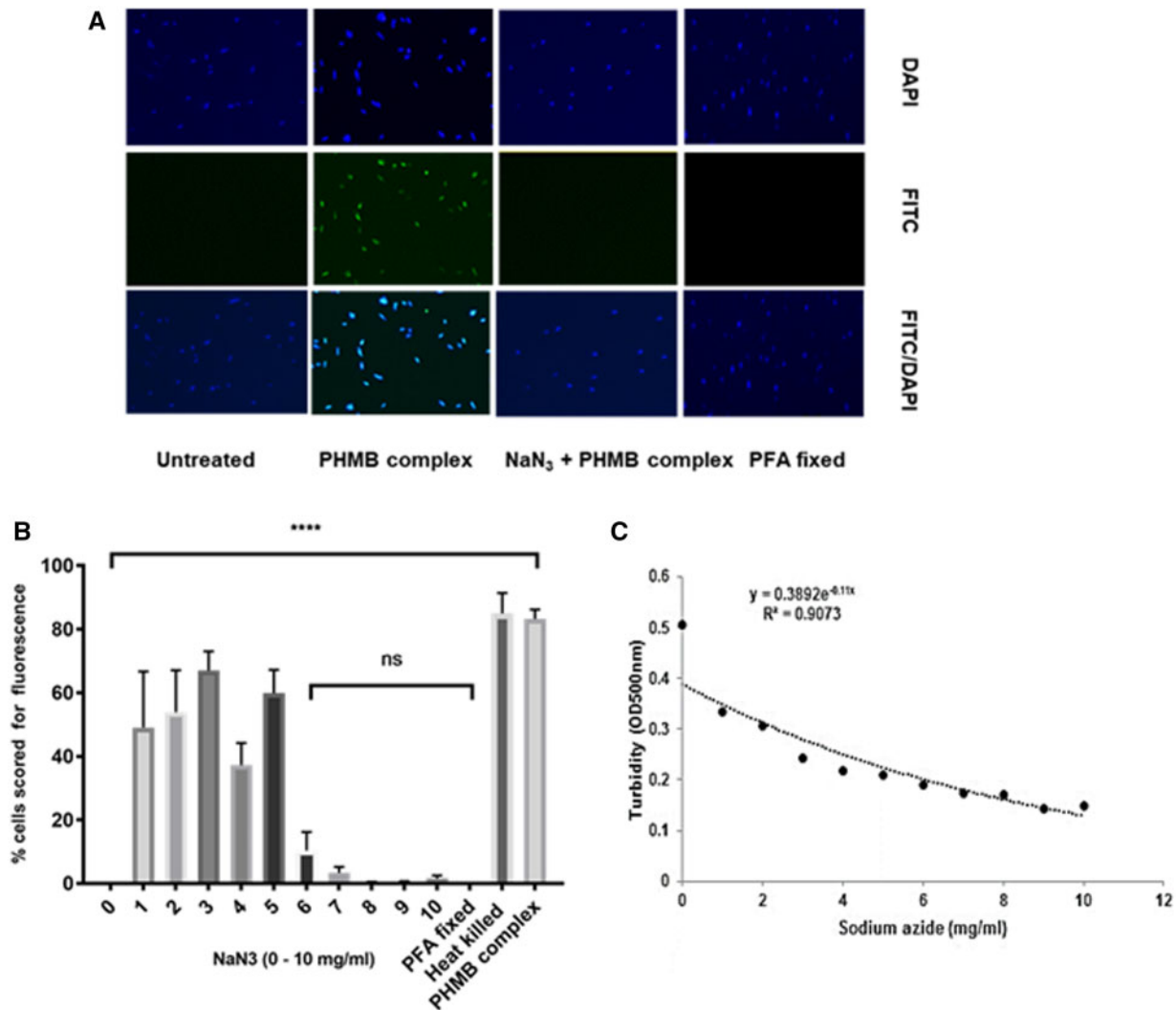


Figure 5: Effects of sodium azide on cell viability and PHMB-oligomer probe delivery. (a) *Salmonella* NK262C cells were first treated with varying concentrations of sodium azide (from concentrations 0–10 mg/ml) and thereafter treated with PHMB-oligomer complex. (b) Quantification of PHMB delivery efficiency into *Salmonella* cells treated with or without NaN₃, PFA and heat. (c) Non-linear regression growth analysis of *Salmonella* cells treated at varying NaN₃ concentrations. Bacterial growths were measured at OD 550 nm. Data are presented as the mean and standard error of the mean for biological replicates ($n = 3$).

Standard FISH versus mFISH

To determine the efficiency of the mFISH method, the protocol was compared with the standard FISH technique. Cell-associated fluorescence was quantified for both procedures. Comparative analysis indicated mFISH performance was equally as efficient as the sFISH ($P > 0.05$). Both methods produced strong fluorescence signals and detection of *Salmonella* (Fig. 7a).

Direct detection of *Salmonella* in artificially contaminated matrices using FISH in combination with PHMB (mFISH)

The mFISH method developed in this study was further tested for the detection of *Salmonella* in artificially contaminated matrices (water and milk). In previous experiments, contaminated milk samples produced little fluorescence and a specific background signal due to interfering milk lipids and proteins. However, further dilution of milk samples (1:10) before mFISH improved detection. No pre-enrichment step was required for

the water and milk, while bacteria detection was achieved in a turnaround time of 5 h (Fig. 7b and c).

Discussion

FISH is a simple technique used for identification of whole-cell bacteria through sequence-specific hybridization of DNA oligomers to complementary target RNA sequences in bacteria. Despite the considerable technological improvement of FISH in past decades, bacterial cell wall permeability to DNA oligomers remains a substantial challenge. For this technique to be useful, DNA oligomers must efficiently pass the stringent bacterial cell barriers. To enhance cell internalization of DNA oligomers, the conventional FISH protocols permeabilize cell membrane barriers using harsh treatments and conditions such as fixation, which involves incubation with PFA [8, 16, 27]. For a possible alternative strategy, we explored using a cation polymer, PHMB (a widely used antimicrobial/disinfection agent) as a carrier molecule. PHMB can interact with oligomers, plasmid DNA, siRNA, proteins and small drug molecules to form nanoparticle

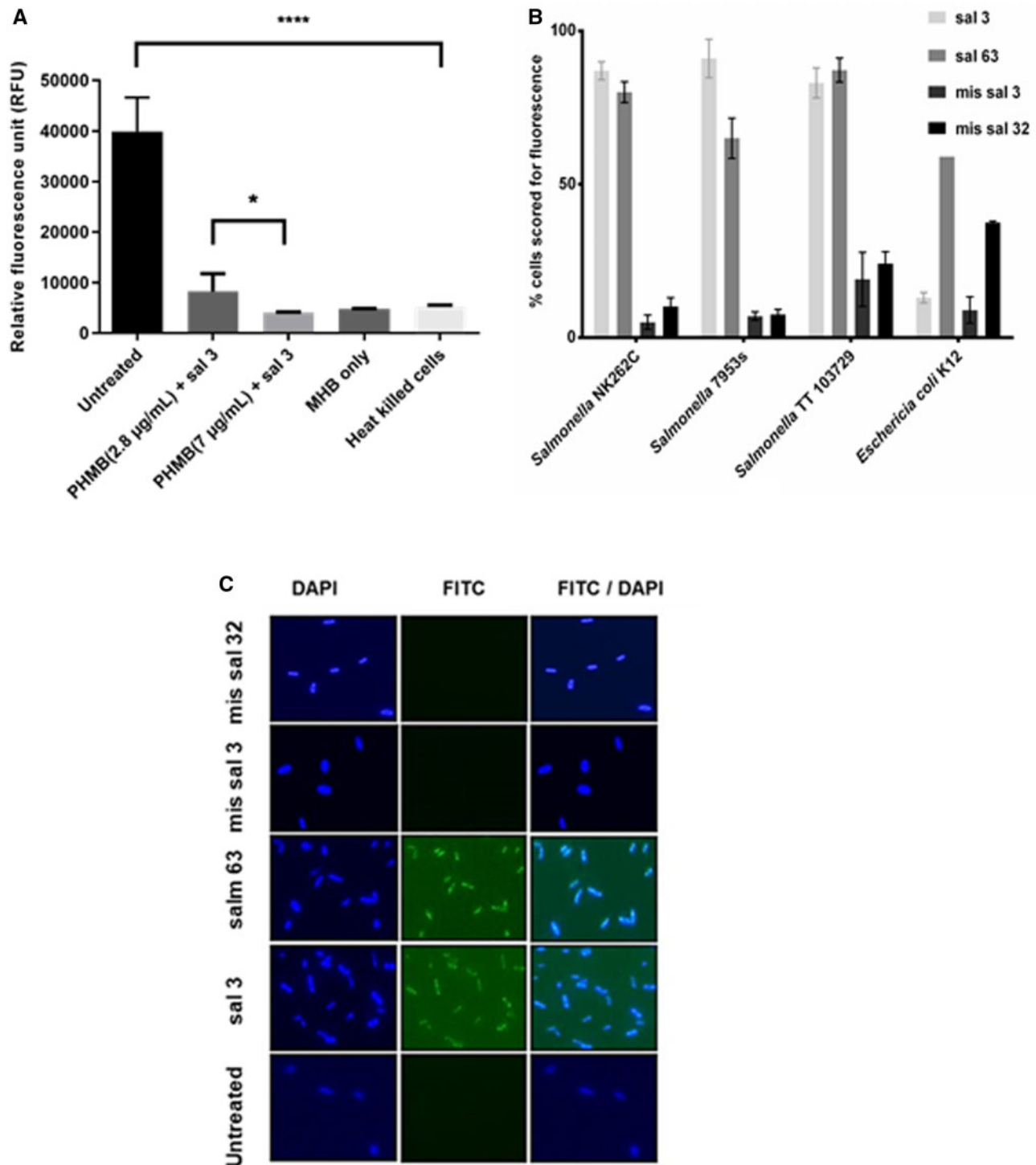


Figure 6: mFISH detection of *Salmonella* NK262C using PHMB for oligomer delivery. (a) Resazurin cell viability assay. (b) Positive and negative control FISH oligomers used for detection of monoculture *Salmonella* bacteria cells. Cell-associated fluorescence post mFISH were quantified and method efficiency estimated. (c) Detection of *Salmonella* by mFISH using PHMB complexed with species-specific oligomers sal 3 and salm 63, and control negative mismatched oligomers mis sal 3 and mis sal 32. *Salmonella* cells were detected as green fluorescent cells when visualized under microscopy with sal 3 and salm 63. Data are presented as the mean and standard error of the mean for biological replicates ($n = 3$).

complex, which can be delivered intracellularly into bacteria, fungi and mammalian cell types [18, 19].

In this study, we reported PHMB-mediated delivery of oligomer into metabolically active (living) bacteria and enhanced rapid detection through a reproducible fixation-free FISH

protocol. PHMB–oligomer complex delivery was achieved into Gram-negative strains of *S. enterica*. The efficient bacterial internalization suggests PHMB as a potential carrier for covalent conjugation of diagnostic oligomers. The PHMB penetration into bacterial cells has been proposed to be attributed to either its

Table 3: Comparing the mFISH method to culture and PCR-based techniques

S/N	mFISH	Culture-based method	PCR-based method
1.	Rapid with results available within 5 h.	A time-consuming process, and laborious. Results may take 2–5 days.	Rapid with a turnaround time of about 24 h
2.	This method is specific and allows visualization of intact or whole bacteria cells using fluorescently labelled oligomers complementary to RNA target sequences	Accompanied by a low level of sensitivity and can fail to identify important pathogens	This method is specific and precisely analyzes the genetic material of pathogens using specific primers
3.	Expensive as it requires expertise and fluorescence microscopy or flow cytometry for cell counting	Relatively cheap method	Expensive because of the equipment and expertise required for detection
4.	The protocol does not require pathogen cultivation or gene amplification	The pathogen of interest has to be cultured	The method requires the amplification of the target DNA for pathogen detection.
5.	Enhances oligomer delivery into living cells and subsequent detection	Useful for the detection of viable bacteria	Detect DNA from dead bacteria cells
6.	Involves fixation-free protocol	Fixation free	Fixation free
7.	The preparations are temporary because fluorescence signals vanish quickly during microscopy	Isolates can be preserved for a longer period	Amplified amplicon signals are long lived
8.	Has the potential to detect more than one target sequence using non-overlapping fluorophores	For pathogen of interest, selective media are used to support growth while inhibiting the growth of other bacteria.	Can also be used for the simultaneous detection of multiple targets in a single reaction using different primer pair sets (multiplex PCR)

amphipathic nature like microbial penetrating peptides or that PHMB creates pores on the cell wall/membrane [19]. PHMB oligomer delivery into actively growing early log phase cells is also reported and supported by past studies which have demonstrated early exponential phase *Salmonella* cells grown within 2 h exhibited unusually high sensitivity to 1-N-phenyl naphthylamine, NPN uptake than mid or late log phase [28–30]. Oligomer delivery into live metabolically active cells observed during microscopy was striking considering the bacteriostatic and bactericidal effects of PHMB [19, 31, 32]. The cell viability assays conducted further indicated metabolic cell activities may be needed for PHMB at low concentrations to facilitate oligomer delivery before bacterial identification. PHMB oligomer delivery optimal efficiency increased with temperature especially in cells grown at 37°C, which is the temperature for optimal enzymatic and metabolic cell activities [33] and further corroborate a related study which showed the need for energy metabolism for PHMB uptake [19]. Additionally, *Salmonella* cells treated with NaN₃ concentrations above 6 mg/ml and with PHMB-oligomer complex displayed little or no delivery. An earlier study demonstrated killing *E. coli* WP2/WP100 cells with NaN₃ at 5 mg/ml in an *in vitro* differential killing study [26]. Since *Salmonella* and *E. coli* are closely related bacteria, we are not astonished by the finding above. Further data generated from this study which monitored bacterial growth at different PHMB concentration treatments indicated noticeable cell viability at PHMB concentration (2.8 µg/ml) than at higher doses. The bactericidal properties of PHMB have been demonstrated with several bacteria species by previous studies [19, 31, 32]. Likewise, bactericidal effect of PHMB on *Salmonella* was observed at concentrations from 6 µg/ml and above in an experiment conducted before deciding to use the lowest concentration of (2.8 µg/ml) that supported delivery into metabolically active bacteria cells.

Aside delivery of oligomers into active *Salmonella* cells, our second objective was to eliminate the fixation step and enhance visualization. The prospect of using PHMB to enhance standard

FISH technique through the promotion of a fixation-free FISH in a single assay and subsequent bacteria detection was achieved. A past study likewise demonstrated an in-solution fixation-free FISH targeting bacterial and archaeal population in sludge samples but by using group-specific oligonucleotide oligomers [34]. The delivery and fixation-free hybridization under a high pH 12 complexation buffer is quite difficult to explain, and a further area of investigation in PHMB studies. Alternatively, PHMB at low concentrations could have destabilized bacterial DNA by weakening the hydrogen bonds with, thus enabling a lower temperature to be used with high stringency [35, 36].

We similarly report the detection of *Salmonella* in artificially contaminated samples using optimized FISH protocols without pre-enrichment or fixation. High temperature and short time post-hybridization protocol were applied to improve specific binding in the milk matrices. Initially, the approach proved unsuccessful in milk samples because of unspecific background signals, which was later addressed by dilution. This limitation was likely associated with the presence inhibitors such as milk fats and proteins, which produced unwanted background signals that interfered with the visualization of the hybridized *Salmonella* cells. In another genotypic method we developed, the treatment and detection of *Salmonella* in artificially contaminated milk sample using species-specific antisense PNAs proved difficult until a clean-up procedure was incorporated to remove inhibitors, improve PNA cellular bioavailability and detection efficiency [37]. Unfortunately, we did not consider artificially contaminating *Salmonella* in fat-free milk to check if the bacterial detection would still be restricted with a lot of background as observed in the undiluted milk sample. Although the mFISH technique is still in its developmental phase with the aim to detect or show the presence of *Salmonella* in water and milk samples, the method was rapid and simple with turnaround of 5 h in relative to the standard culture-based methods (Table 3). For this study, *Salmonella* was detected in artificially contaminated water and milk samples at 1×10^6 and 10^5 cfu/ml.

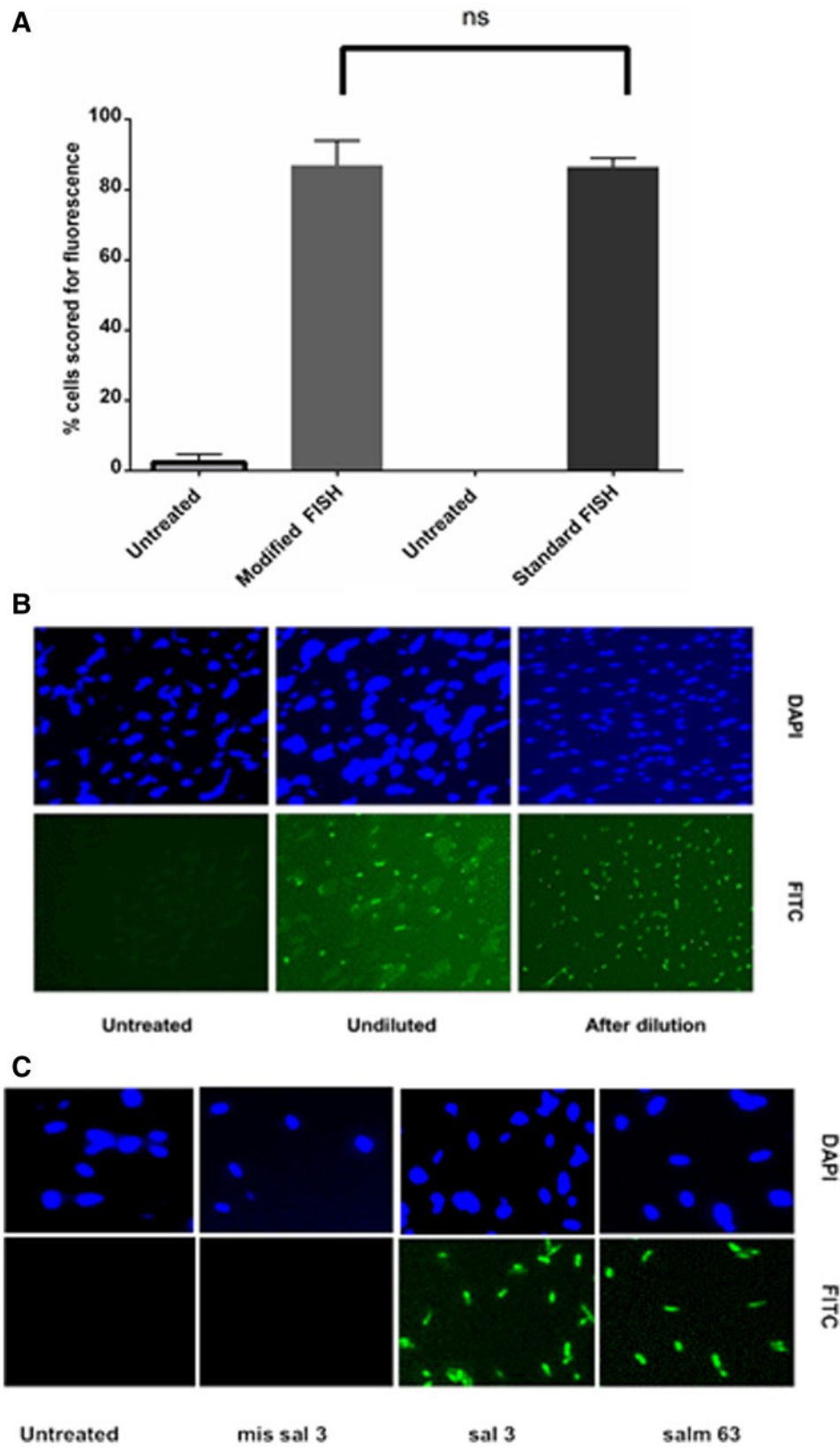


Figure 7: Detection of *Salmonella* NK262C strain using a mFISH method. (a) Comparison of standard FISH and PHMB modified methods was conducted and efficiency quantified as cell-associated fluorescence using volocity 6.3. (b) *Salmonella* detection in artificially contaminated milk sample after a 10-fold dilution. No pre-enrichment step was involved. Detection of bacteria in undiluted milk samples was occluded by background signals (middle panel). (c) *Salmonella* detection in artificially contaminated water without pre-enrichment using mFISH. Data are presented as the mean and standard error of the mean for biological replicates ($n = 3$).

Previous-related studies reported a detection limit of $(2 \times 10^7 \pm 5 \times 10^6)$ cfu/ml in milk after an 8-h enrichment step using PNA-FISH [5]. The lowest concentration at which the mFISH could detect *Salmonella* in food samples was not considered in this study but in future studies. Similarly, this technology was applied to only Gram negative bacteria. Previous studies have demonstrated the successful uptake of PHMB in Gram positive bacteria (*Staphylococcus aureus*) [19, 31], and acid fast *Mycobacterium smegmatis* [19], and suggests to us the fixation-free FISH technology following adequate optimization has the potential to detect Gram-positive bacteria and overcome challenges encountered with the standard FISH in the detection of these group of organisms.

Conclusion

There were two main findings in this study. First, PHMB promoted oligomer delivery into viable bacteria cells. Second, PHMB supported a fixation-free FISH protocol for *Salmonella* detection. The mFISH method developed in this study provides an easy and efficient approach for rapid bacteria detection in monoculture strains, water and milk matrices, without a pre-enrichment step. Our findings suggested that PHMB is potentially an efficient delivery and hybridization agent, and enhanced standard FISH method by eliminating the conventional fixation steps, and have future application in rapid detection of *Salmonella* and establishing a fluid phase test for flow cytometric detection of *Salmonella*. Also, the mFISH method may be a useful tool in environmental research especially, in microbial population studies. Extension of the approach to the detection of Gram-positive bacteria and fungi may be possible, but would likely require method adjustments. Moreover, as this method is modified, further work will be to test its application on a wider range of *Salmonella* strains and non-*Salmonella*-related bacteria, as well as promote its potential practical application in real samples.

Supplementary data

Supplementary data are available at *Biology Methods and Protocols* online.

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Authors' contributions

A.O.: formal analysis, investigation and writing—original draft preparation. L.G.: conceptualization, writing—review and editing and supervision. A.O.O. and L.G.: methodology and read and agreed to the published version of the manuscript.

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