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Research article

The development of species-specific antisense peptide nucleic acid method for the treatment and detection of viable Salmonella

Oluwawemimo O. Adebowale [1,2,*], Shan Goh [1,3], Liam Good [1]

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ABSTRACT

Genotypic based detection methods using specific target sites in the pathogen genome can complement phenotypic identification. We report the development of species-specific antisense peptide nucleic acid (PNA) combined with selective and differential enrichment growth conditions for *Salmonella* treatment and detection. An antisense PNA oligomer targeting the *Salmonella ftsZ* gene and conjugated with a cell-penetrating peptide ($(KFF)_3K$) was exploited to probe bacteria cultured in three different growth media (Muller Hinton broth (MHB), Rappaport-Vassiliadis Soya Peptone Broth (RVS, Oxoid), and in-house modified Rappaport-Vassiliadis Soya Peptone Broths (mRVSs). Also, water and milk artificially contaminated with bacteria were probed. Antisense PNA provided detectable changes in *Salmonella* growth and morphology in all media and artificially contaminated matrices except RVS. *Salmonella* was detected as elongated cells. On the contrary, treated *Escherichia coli* did not elongate, providing evidence of differentiation and selectivity for *Salmonella*. Similarly, *Salmonella* probed with mismatched PNAs did not elongate. Antisense oligomers targeted *ftsZ* mRNA in combination with selective growth conditions can provide a detection strategy for viable *Salmonella in* a single reaction, and act as a potential tool for bacteria detection in real food and environmental samples.

1. Introduction

Salmonella is a Gram-negative flagellated rod-shaped bacterium, and one of the most critical enteric infectious disease burdens worldwide [1]. *Salmonella* especially Nontyphoidal strains constitute a significant cause of diarrheal diseases in humans globally particularly in developing countries, which is estimated to cause 93 million enteric infections and 155,000 diarrheal deaths annually [2]. The bacterium is among the most commonly isolated foodborne pathogens and remains near the top of the list of infectious pathogens of public health concern among international food standards agencies, food retailers, and consumers. Rapid identification of the bacteria promotes early warning and responses to disease outbreaks and effective epidemiological surveillance [3].

Improving diagnostic methods is crucial for the mitigation of foodborne illness and deaths caused by *Salmonella* spp, [4]. The detection and reporting of *Salmonella* still largely relies on culture-based methods, which is the acceptable golden standard for pathogen identification worldwide [5]. The conventional culture method provides a slower turnaround time for detection and lower sensitivity and specificity [5 6]. The culture processes are time-intensive and laborious and take between 3 and 5 days including selective enrichment protocol for positive result outcomes. The lack of promptness may not be convenient especially where early interventions or warnings are required to control pathogen transmissions during outbreaks. Due to the limitations confronted by culture methods, development of newer sequence-based diagnostic methods that exploit stable genotypic characteristics of specific pathogens are vital and constantly evolving [7]. More rapid techniques such as polymerase chain reaction (PCR), quantitative PCR, enzyme-linked immunosorbent assay (ELISA), and fluorescence in situ hybridization (FISH) have been developed to overcome challenges associated with culture methods, regarding rapidity [8]. However, these molecular methods are limited by problems that prevent them from fully replacing traditional culture methods. For instance, some problems associated with molecular methods are the inability to detect and discriminate viable/non-viable bacteria, susceptibility inhibitors (such as proteins, fats, salts) present in samples, cross-reactivity, and low sensitivity. Also,

* *Corresponding author.*
E-mail address: adebowaleoluwawemimo1@gmail.com (O.O. Adebowale).
[1] Current address: Department of Pathobiology and Population Sciences, Royal Veterinary College, London, United Kingdom.
[2] Current address: Department of Veterinary Public Health and Preventive Medicine, College of Veterinary Medicine, Federal University of Agriculture Abeokuta, Ogun State, Nigeria.
[3] Current address: Department of Biological and Environmental Sciences, School of Life and Medical Sciences, University of Hertfordshire, United Kingdom.

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past studies have shown culture methods especially the enrichment steps in combination with molecular methods enhance assay sensitivity by eliminating problems such as the low numbers of bacteria and the presence of inhibitory substances in certain samples types [3].

Peptide nucleic acid (PNA) is a pseudopeptide DNA mimic and the novel properties first discovered in 1991 at the University of Copenhagen in Demark [5, 9]. PNA has desirable properties for developing antisense agents that inhibit gene expression *in vitro* through sequence-specific rRNA binding [10, 11]. The standard PNA sequences offer specific unique advantages over DNA oligomers. PNA has increased thermal stability, resistance to degradation by enzymes, stability in acidic environments and superior specific sequence hybridization efficiency [10, 12]. Based on these qualities, PNAs have found its full application in antigene and antisense therapeutic and research developments [13, 14, 15, 16]. The standard PNA oligomers in combination with genotypic methods such as fluorescence *in situ* hybridization (PNA-FISH) have been reported for bacterial diagnostics, but still limited in its ability to detect live/viable bacteria cells [17, 18]. Several past studies have shown that antisense *ftsZ* PNA resulted in cell elongation of *Escherichia coli*, *Salmonella* and methicillin-resistant *Staphylococcus aureus* making this a promising antibacterial target [12, 14, 15, 16, 19, 20]. However, its strategic application in bacterial diagnostics has not been reported.

In this preliminary study, we tested the hypothesis that the antisense strategy can be exploited to develop a rapid method for detection of viable *Salmonella*. The specific targeting of antisense PNA to *Salmonella ftsZ* mRNA could selectively suppress the bacteria cell division, causing phenotypic modifications in selective and differential growth media. We report, for the first time, that antisense peptide- PNAs can form the basis for both the genotypic and phenotypic assessment and detection of *Salmonella* in a single assay.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The bacteria strains used in this study are listed in Table 1. Bacteria were maintained in 30% glycerol stock at -80 °C until use. Colonies of *Salmonella enterica* Typhimurium LT2, *Salmonella enterica* Typhimurium 7953S, *Salmonella enterica* Typhimurium TT10329, and *Escherichia coli* K12 were grown on Luria-Bertani agar (LB; Oxoid, UK) plates at 37 °C for 16–18 h. Likewise, *Salmonella enterica* Typhimurium LT2 pGlo (AmpR) and *Salmonella enterica* Typhimurium pdsRed (AmpR) were grown on LB agar plates supplemented with ampicillin (1 μg/mL). Before antisense PNA treatment, PCR assays to validate bacterial species identities according to Pathmanathan et al., 2003 [21] were performed.

2.2. Antisense PNA oligomer

The anti- *Salmonella ftsZ* peptide nucleic acids were designed according to Good et al 1998 [13] and Mondhe et al., 2014 [19]. The -5 to +5 start codon region of the translation reduction region (TIR) of *ftsZ* mRNA in *Salmonella* Typhimurium with two base pair mismatches to the TIR of *Escherichia coli* was targeted to enhance species discrimination. Scrambled PNA mismatched sequences 404 and 405, non-specific for *Salmonella* were used as negative controls. To enhance the delivery of PNA through stringent bacterial cell walls, antisense PNAs was conjugated covalently by the ethylene glycol linker (8-amino-3, 6-dioxaoctanoic acid) to the peptide $(KFF)_3K$ attached to 5' end of oligomers. The conjugated PNA was produced by Panagene, Korea. Table 2 shows the details of the antisense peptide nucleic acid oligomers used for this study.

2.3. Preparation of selective media

Commercial selective enrichment medium, Rappaport-Vassiliadis Soya broth (RVS; Oxoid, UK) was prepared according to the manufacturer's guidelines. The In – house modified RVS low salt media (mRVSs) was freshly prepared by adjusting salt concentrations of the full basal strength of commercial RVS. The mRVSs media comprised of 0.96 mM, 0.72 mM, 0.48 mM sodium chloride (NaCl), and 0.70 mM magnesium chloride ($MgCl_2$). The mRVSs media were prepared in 100 mL volumes, pH adjusted to 5.2 ± 2 and autoclaved at 121 °C for 15 min.

2.4. Non-selective and selective growth conditions for antisense PNA assay

Salmonella and *E. coli* K12 strains were standardized at OD 600 nm readings to approximately 10^6 cfu/mL. Cultures in 20 μL volumes were deposited in ultra-low - bind polystyrene 96 - well assay plate (Costar, UK) containing 170 μL of MHB and anti-*Salmonella ftsZ* PNA at 0-1 μM to a final volume of 200 μL per well. The plate was incubated in a plate reader (Spectramax® 340 PC) at 37 °C with agitation and growth monitored at OD 550 nm at every 5 min for 6 and 16 h. *Salmonella enterica* Typhimurium LT2 JR501, *Salmonella enterica* Typhimurium LT2 JR 501 PGLO (AmpR), and *Salmonella enterica* Typhimurium LT2 JR 501 pdsRed (AmpR) were used for this investigation.

The assay was similarly performed for *Salmonella enterica* Typhimurium 7953S and *Salmonella enterica* Typhimurium TT 103729 under selective growth conditions. The *Salmonella* strains (10^6 cfu/mL) were grown in commercial Rappaport-Vassiliadis Soya broth and in-house low salt modified RVS media (mRVSs low salt media - 0.70 mM $MgCl_2$) and treated with anti-*Salmonella ftsZ* at 0–3 μM. PNA oligomers 404 and 405, and *E. coli* K12 were included as the negative controls.

Table 1. Description of bacterial strains used for preliminary screening for antisense PNA sequence specificity and treatment.

Strains	Genotypic characteristics	Source	Purpose
Salmonella enterica serovar Typhimurium LT2 JR501	R^-m^+ for converting plasmids to *Salmonella* compatibility strain[1]	SGSC 1539[2]	Preliminary screening for antisense PNA sequence specificity and treatment assays
Salmonella enterica serovar TyphimuriumLT2 JR 501 PGLO (AmpR)	Reporter gene GFP (Aequorea Victoria), IPTG inducible, ampicillin-resistant plasmids.		
Salmonella enterica serovar Typhimurium *LT*2 JR 501pdsRed (AmpR)	Reporter gene RFP (variant of Discosoma sp), IPTG inducible, ampicillin-resistant plasmids		
Salmonella enterica serovar Typhimurium7953S	Virulent phoPQ: Tn10 mutant of serovar Typhimurium LT2	SGSC 1539[2]	Antisense PNA assays
Salmonella enterica serovar TyphimuriumTT103729	n/a		
Escherichia coli K12	strain		

[1] Restriction deficient, modification proficient cloning strain of *Salmonella*.
[2] SGSC, *Salmonella* Genetic Stock Centre. University of Calgary, Canada.

Table 2. Details of the antisense peptide nucleic oligomer sequences used for this study.

PNA oligomers	Sequence	Target gene	Target site	MW	Reference
anti-*Salmonella ftsZ*	$(KFF)_3$K-eg-aac ata atc t	*ftsZ*	-5 to +5	4235.5	This study
PNA 404	$(KFF)_3$K-eg-caa atatct at	-	N/A	4235.5	This study
PNA 405	$(KFF)_3$K-eg-aac taa ctt ta	-	N/A	4235.5	This study

2.5. Artificially contaminated water and detection of Salmonella using antisense PNA

Water confirmed free of *Salmonella* by culture methods was used for the seeding experiments. Ten-fold serial dilutions of *Salmonella* 7953S and TT 103279 culture broths were prepared by adding 1 mL volumes to 9 mL sterile water at 10^6 cfu/mL final concentration of *Salmonella*. Volumes of 20 μL from each dilution series were probed with antisense PNA under selective growth conditions as described previously. Non – artificially contaminated water samples, water artificially contaminated with *E. coli* K12, were used as negative controls.

2.6. Artificially contaminated milk and detection of Salmonella using antisense PNA

Milk confirmed free of *Salmonella* by culture method was used for the seeding experiments. Milk was reconstituted by mixing 2.5 g of skimmed milk powder (Sainsbury, UK) to 22.5 mL sterile distilled water. Reconstituted milk was then artificially contaminated with overnight *Salmonella* 7953S (10^6 cfu/mL) broth culture grown in MHB, and incubated at room temperature for 5 min. Artificially contaminated milk was pelleted by centrifugation at 4,000 rpm for 10 min and resuspended in 1 mL sterile water and desalted using Zeba spin desalting columns (7KMW cut-off; Pierce Technology, USA) according to manufacturer recommendations. This is a high-performance resin that offers exceptional desalting for protein samples. Desalted contaminated milk (500 μL) was used for the antisense treatment under selective growth conditions as described previously. Non-artificially contaminated milk and artificially contaminated *E. coli* milk were used as negative controls.

2.7. Microscopy

Cells were prepared for fluorescence imaging. Cells harvested were washed twice in (1 mL), resuspended in DAPI (1 μM, Invitrogen, UK), and incubated in a dark chamber for 15 min at room temperature. Aliquots of 3–5 μL of the resuspended cells were deposited on slides, covered with glass slips and visualized with the blue filter (excitation wavelength 340–380 nm and emission wavelengths 450–490 nm) using a LEICA DM4000B fluorescence microscope with Zeiss AxioVision software.

2.8. Bacterial growth and enumeration in RVS and mRVSs media

For the selective assay, volumes of 20 μL of standardized *Salmonella* 7953S and *E. coli* K12 culture broths (at OD600nm) were mixed thoroughly and added to RVS and mRVSs media to final volumes of 200 μL. The 96 – well plate was then incubated at 37 °C for 16 h with regular monitoring. After that, cells were washed, resuspended in 200 μL volumes of PBS and serial dilutions at 10^1- 10^5 prepared. A volume of 100 μL from each dilution series was spread plated on MacConkey agar (MA, differential medium) and incubated at 37 °C for 24 h.

The enrichment assay was carried out by growing *Salmonella* 7953S in both RVS and mRVSs at 37 °C for 16 h, and growth monitored every 5 min. Later, cells were harvested, rinsed and resuspended in a 200 μL volume of PBS. Serial dilutions as described previously were prepared and plated on Luria-Bertani agar (LB; non-differential/universal media). The average bacteria count in both assays were determined and colony-forming unit estimated.

2.9. Statistical analysis

Data generated from experiments (optical density and bacterial enumeration) were plotted as mean ± SEM for two or three replicates. 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 was used (2-way-ANOVA) for the comparison of means of more than two independent groups with 2 or more outcomes. Post hoc analysis comparisons were also performed. All analyses were conducted using statistical packages Prism 6, Version 6.0 (GraphPad Prism 6.0, San Diego, CA, United States). Differences were scored statistically significant if the p-value was ≤ 0.05. Illustrations were performed by GraphPadPrism 6®.

3. Results

3.1. Antisense PNA mediated species-specific morphological shift and bacterial detection in non-selective MHB

To determine the specificity of antisense PNA oligomer (anti – *Salmonella ftsZ*) in the detection of *Salmonella*, three strains of the bacteria and closely related *E. coli* K12 were used. *Salmonella enterica* Typhimurium LT2 JR 501, *Salmonella enterica* Typhimurium LT2 JR 501 PGLO (AmpR), and *Salmonella enterica* Typhimurium LT2 JR 501 pdsRed (AmpR) used for preliminary investigations exhibited reduced growth rates post-treatment with oligomer. Growth OD measurements showed PNA concentrations 0.2, 0.5 and 1.0 μM visibly reduced *Salmonella enterica* Typhimurium LT2 JR 501 and *Salmonella Enterica* Typhimurium LT2 JR 501 PGLO (AmpR) by 17, 76 and 93%, and 36, 85, and 96% correspondingly compared to untreated cultures (Figure 1a). Growth reductions of 29, 61, 90, and 100% were estimated for *Salmonella Enterica Typhimurium* LT2 JR 501 pdsRed (AmpR) at PNA treatments 0.1, 0.2, 0.5 and 1.0 μM respectively. Growth inhibitory effect of PNA with phenotypic shift was observed in only *Salmonella* strains but not closely related *E. coli* K12.

Furthermore, *Salmonella enterica* Typhimurium LT2 JR501, *Salmonella enterica* Typhimurium LT2 JR 501 PGLO (AmpR), and *Salmonella enterica* Typhimurium LT2 JR 501 pdsRed (AmpR) exhibited detectable elongation after 16 hour incubation. However, a morphological modification was not observed for *E. coli* K12 (Figure 1 b). Our findings indicated the antisense PNA treatment was species-specific and ensued phenotypic changes, which may serve as an important biological marker for bacterial detection.

3.2. Antisense PNA enabled rapid detection of Salmonella growth phenotypes in non-selective media

Antisense PNA treatments were conducted to test the ability to rapidly detect *Salmonella* enterica Typhimurium 7953S and *Salmonella* enterica Typhimurium TT 103729. Aliquots of probed cells were harvested at 6 h, and cell morphology examined. *Salmonella* was observed as filamentous cells at 6 h at all treatment PNA concentrations but not in untreated *Salmonella* cells and antisense PNA-treated *E. coli* K12 retained normal cell morphology (Figure 2).

Cultures of *Salmonella* strains treated with anti-*Salmonella ftsZ* PNA oligomer at indicated concentrations. Untreated cells and PNA treated *Escherichia coli* K12 were used as negative controls. Species-specific phenotypic alterations were observed in *Salmonella* cells indicating

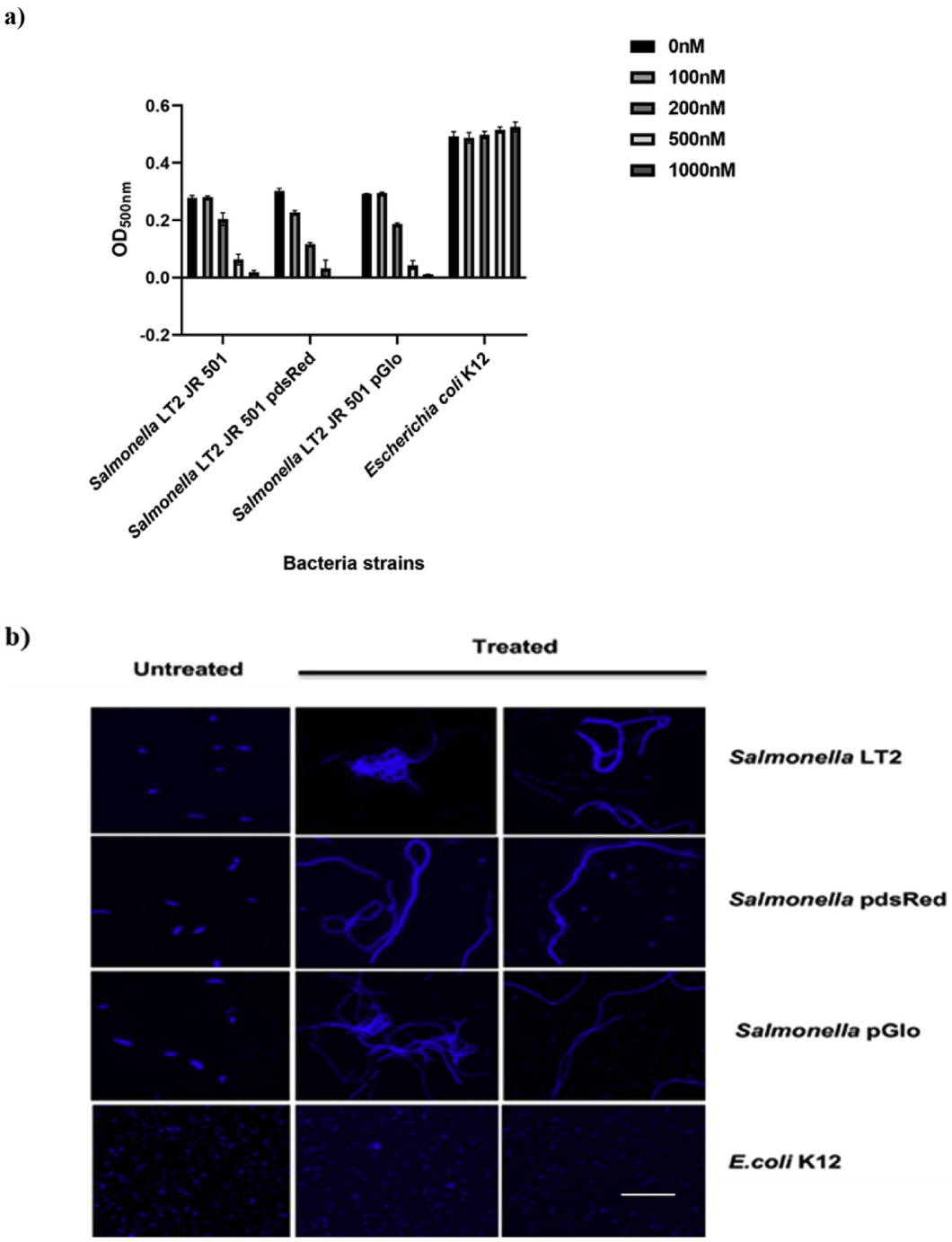

Figure 1. Effect of anti-*ftsZ Salmonella* oligomer on *Salmonella* and *E. coli* strains. (a) Effect of varying concentrations of antisense PNA *ftsZ* oligomer (0–1000 nM) on bacteria cell culture growths. (b) Fluorescence microscopy of the assay (antisense *ftsZ* PNA at 1000 nM). Cells were stained with DAPI and detected as filaments or elongated cells indicating antisense expression of the *ftsZ* gene. Visualization was carried out under blue filter cubes at excitation wavelength 340–380 nm and emission wavelengths 450–490 nm.

silencing of *ftsZ* expression. Bacteria were detected as elongated cells after 6 h post incubation.

3.3. Effects of temperature on Salmonella morphology in RVS and mRVSs

To determine an optimal temperature for antisense PNA assays under selective growth conditions, *Salmonella* Typhimurium 7953S and *E. coli* K12 strains were cultured in the presence of antisense PNA at 37 °C and 42 °C. *Salmonella* treated with antisense PNA displayed cell elongation in both RVS and mRVSs at 42 °C. On the other hand, at 37 °C, cells showed elongation only in mRVSs while normal morphology was retained in RVS media. Antisense PNA-treated *E. coli* K12 grown in RVS and mRVSs at both temperatures retained normal cell morphology. To ensure that the growth effects were PNA-mediated, growth assays at 37 and 42 °C without PNA were included. *Salmonella* cells were still observed elongated in RVS and mRVSs at 42 °C, but not 37 °C. Therefore, 37 °C was used as the optimal temperature for the assay under selective enrichment conditions.

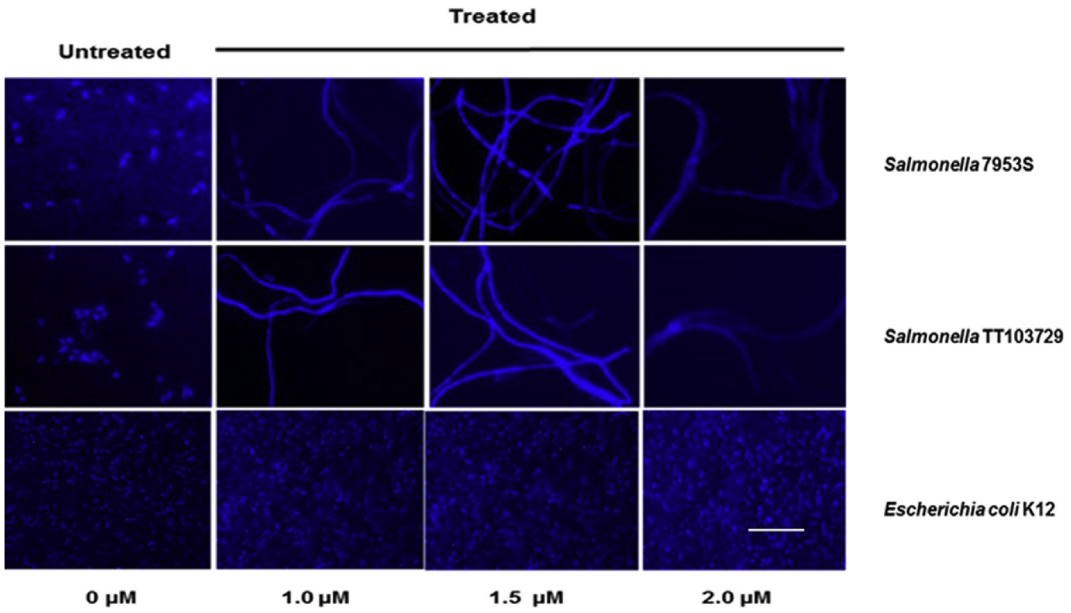

Figure 2. Antisense PNA Oligomer and detection of *Salmonella* in non-selective conditions.

3.4. Antisense PNA in combination with selective enrichment growth conditions supported Salmonella detection

We hypothesized that selective enrichment medium in combination with antisense PNA treatment could enhance selective identification of *Salmonella*. RVS is the recommended commercial selective enrichment medium for the isolation of *Salmonellae* (ISO 6579:2002), hence chosen for these experiments. We observed *Salmonella* cells treated in this medium displayed no growth reduction or phenotypic changes even at higher PNA concentration treatments (>3.0 μM). Similarly, non-targeted *E. coli* showed no reduced growth or morphological modifications (Figure 3).

To determine whether salt concentrations in the RVS growth medium were inhibiting factor to antisense PNA performance, we varied NaCl and $MgCl_2$ concentrations. With these modifications included, *Salmonella* growth was reduced with evidence of phenotypic alteration - from regular rod-shaped to filamentous. The morphological shifts observed occurred in all media; nevertheless, reproducible in mRVSs media 0.70 mM $MgCl_2$ at PNA concentrations 2.5 and 3.0 μM. Cells were detected as early as 6 h after incubation. Non-target *E. coli* K12 remained unaffected under the same growth conditions. *Salmonella* and *E. coli* strains treated with mismatched PNA oligomers 404 and 405 indicated no phenotypic changes. The study findings showed to detect *Salmonella* using the antisense PNA, salt adjusted RVS is required.

3.5. Modified RVS low salt (mRVSs) medium retained selectivity and enrichment qualities for Salmonella growth

The growth of both *Salmonella* and *E. coli* strains in the media types were compared. *E. coli* but not *Salmonella* growth was inhibited in both mRVSs and RVS media ($p < 0.0001$; Figure 4a). To further compare enrichment quality of mRVSs with standard commercial RVS, growth enrichment and viable counts of *Salmonella* 7953S in mRVSs and RVS after 16 hour incubation were carried out. Average bacterial counts and the cfu/mL were estimated for RVS, mRVSs-0.96mM NaCl, mRVSs-0.72 mM NaCl, mRVSs- 0.48mM NaCl and mRVSs-0.70 mM $MgCl_2$ media as follows respectively: 137 (1.4×10^9), 272 (2.7×10^9), 397 ($4.0 \times 10^{9)}$, 162 ($1,6 \times 10^{9)}$, and 216 (2.2×10^9). Comparative analysis results indicated no significant difference ($p = 0.4490$) in average bacterial colony counts determined for various media used in this study. Hence, in-house modified RVS was comparable to commercial RVS in the enrichment of *Salmonella* cultures (Figure 4b).

3.6. Salmonella detected in artificially contaminated water

The detection of *Salmonella* in artificially contaminated water samples was conducted. Viable *Salmonella* cells were elongated in spiked samples (at 10^6 cfu/mL). On the other hand, cell elongation was not observed in the negative controls (Figure 5).

Water samples artificially contaminated with *Salmonella* were grown in selective mRVSs conditions. Bacteria were detected as elongated cells after 16 hour incubation. In contrast, untreated samples, and spiked water probed with negative control PNA 404, and PNA 405 at concentration 3.5 μM retained normal rod-shaped morphology.

3.7. Detection of viable Salmonella in artificially contaminated milk

Milk was artificially contaminated to detect *Salmonella* in a more complex matrix. Preliminary assays provided no desired outcome due to inhibitors present in milk samples. These reduced the efficiency of antisense PNA to detect *Salmonella* directly in samples. However, a clean-up and desalting phase before treatment were performed (Figure 6). No cell elongations were apparent in uncontaminated and contaminated samples treated with mismatched negative control PNA oligomers 404 and 405. Overall, the method performed well in milk only after removal of PNA inhibitors, which may have enhanced bioavailability of PNA to target mRNA site, and detected viable *Salmonella* in milk.

Antisense PNA treated *Salmonella* (10^6 cfu/mL) in artificially contaminated milk samples was detected as elongated cells detected after post sample clean up and desalting. Higher concentrations of anti-*Salmonella ftsZ* PNA probe (3.5 μM) were required to produce detectable signals. No elongation in *Salmonella* cells was observed in milk samples probed with negative control PNA oligomers 404 and 405.

4. Discussion

Salmonellosis is one of the most prevalent infections posing a serious health risk [22]. *Salmonella enterica* is the most studied based on the role in animal and human infections, with both typhoidal and non-typhoidal infections more prevalent in humans in the developing countries [23].

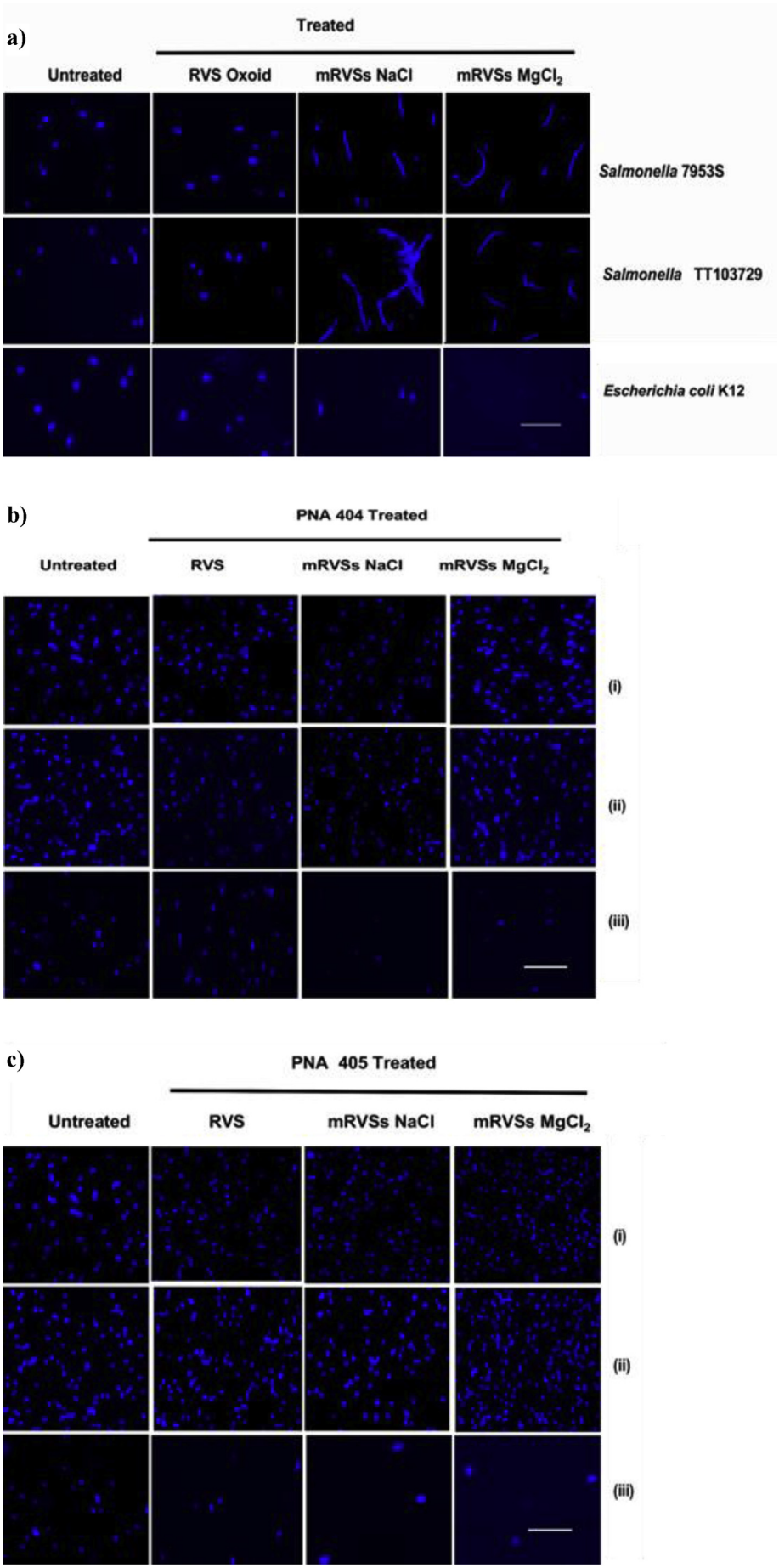

Figure 3. Species-specific detection of *Salmonella* by Antisense PNA *probe* in combination with selective growth conditions. (a) Cultures of *Salmonella* strains probed with antisense *ftsz* PNA were detected as elongated or filamentous cells post 6 h incubation in salt adjusted selective RVS media. (b, c) Mismatched oligomers 404 and 405 used for *Salmonella* strains 7953S and TT 103729 (i and ii respectively) detection produced no detectable phenotypic modifications and for *Escherichia coli* K12 (iii).

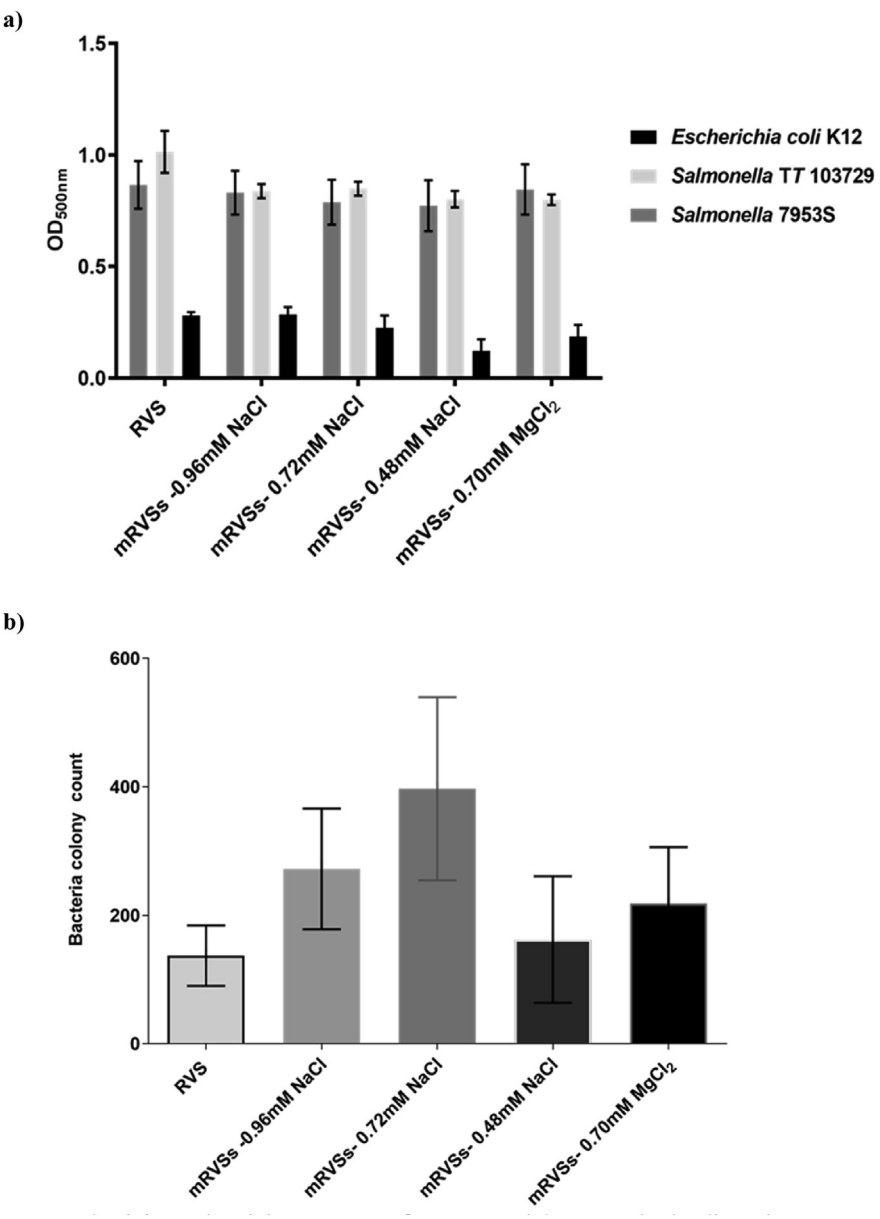

Figure 4. Selectivity and enrichment assays for commercial RVS and salt adjusted mRVSs media. (a) *Salmonella* and *E. coli* cultures grown in commercial RVS and salt adjusted mRVSs media. Both bacteria were incubated for 16 h at 37 °C and growth monitored every 5 min. All media tested yielded good growth of *Salmonella* strains but reduced *E. coli* K12, indicating significant selectivity ($p < 0.001$) for *Salmonella*. (b) Cultures of *Salmonella* were grown in various RVS broths for 16 h, plated onto LB agar plates. The bacteria viable count and cfu/mL were estimated.

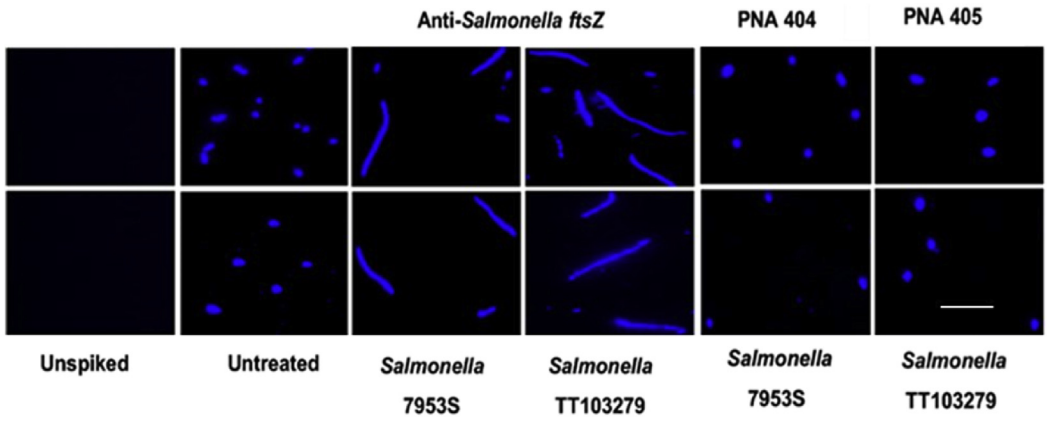

Figure 5. *Salmonella* detection in artificially contaminated water after 3.5 μM antisense PNA treatment.

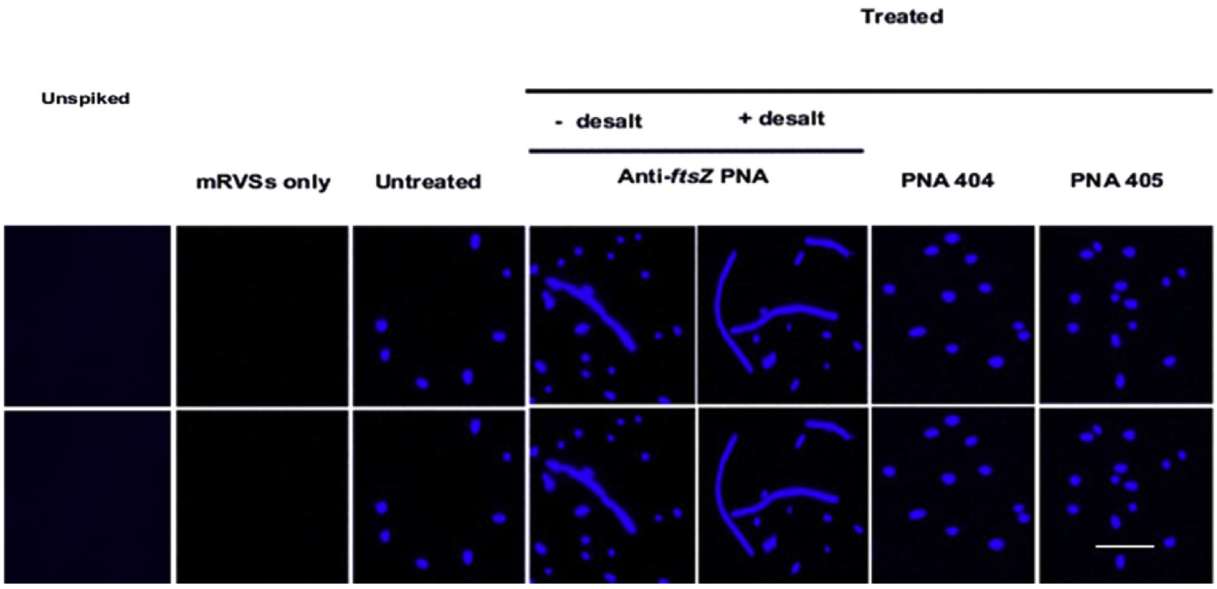

Figure 6. *Salmonella* detection in artificially contaminated milk.

Meanwhile, in the high-income countries, non-typhoidal bacteria are more common and regularly associated with foodborne disease outbreaks. Salmonellosis especially caused by non-typhoidal *Salmonella* group is estimated to cause 93 million enteric infections and 155,000 diarrheal deaths in humans each year globally [24].

In this study, we tested the practicability of using antisense PNA in combination with selective enrichment steps for specific detection of viable *Salmonella*. The *ftsZ* gene is a sensitive target for peptide-PNA mediated growth inhibition, so was chosen in *Salmonella* for this study as a gene knockdown and reduction in the protein expression leads to visible cell elongation [11, 12, 14, 16, 19, 25]. This approach could serve as a potential biomarker for the rapid detection of a broad range of viable bacterial pathogens in a single reaction. Its combination with selective enrichment conditions could provide promptness for pathogen early detection when compared with the use of culture methods only. Antisense peptide nucleic acids have been shown to hold a promising use in gene therapy [26] and antimicrobial studies [11, 13, 19, 27, 28], though its application in microbial diagnostics is limited.

Detectable shifts in morphology from normal rod-shaped to elongated filamentous cells were observed for *Salmonella enterica* Typhimurium LT2 JR501, *Salmonella enterica* Typhimurium LT2 JR 501 PGLO (AmpR), *Salmonella enterica* Typhimurium LT2 JR 501 pdsRed (AmpR) under non-selective growth conditions post 6 hour treatment. In contrast, untreated *Salmonella* and non-target closely related *Escherichia coli* strains did not elongate. Incorporating a 2-base pair mismatch demonstrated the capability of antisense PNA sequence to specifically inhibit translation of the *ftsZ* gene in *Salmonella* but not *E. coli.* This finding also supports observations from other authors who have reported PNA sequences easily and efficiently distinguish bacterial species by single mismatch [20, 29].

Although higher concentrations of PNA (1.0–2.0 μM) were required to detect strains of *Salmonella enterica* Typhimurium 7953S and *Salmonella enterica* Typhimurium TT 103729, identification was achieved after a 6 hour incubation. However, these results were still consistent with previous antisense antimicrobial studies, which demonstrated lower nanomolar or micromolar concentrations of antisense PNA were needed to produce significant down-regulation of essential functional gene expressions in *E. coli* AS19 and D22 transformants (β-galactosidase and β-lactamase) than the wild strains [13, 15]. Similarly, a previous study demonstrated that species selective inhibition of *Salmonella* Typhimurium in mixed culture with DsRed labelled *E. coli* grown in MH broth was achieved at higher PNA concentrations >2.0 μM (same PNA sequence was used in this study) [19]. The higher concentrations of antisense PNA needed for detection of *Salmonella* strains may be associated with differences in species or strains susceptibilities to the PNA uptake.

Additionally, antisense PNA treatment in combination with selective growth conditions further supported differential and selective detection of *Salmonella* in low salt modified RVS media. Surprisingly, within the PNA concentration range used, this method was not selective in ISO recommended RVS medium. The inability to detect *Salmonella* in RVS medium may be explained by the presence of high concentrations of salts, which may have served as inhibitors for PNA delivery to the cell through electrostatic shielding at the cell surface. Excessive $MgCl_2$ (>1 mM) have also been demonstrated to significantly inhibit peptide-PNA bioavailability to the target sequence [9]. The level of $MgCl_2$ concentration in the standard commercial RVS broth was much higher than 1mM and explains why RVS growth conditions would not support antisense detection of bacteria. Hence, the necessity to adjust salt concentrations ($MgCl_2$ and NaCl) to improve probe detection efficiency. The selectivity and enrichment qualities of salt adjusted RVS media were investigated and found comparable with commercial RVS, which indicated the low salt RVS prepared in-house retains the abilities to enrich and select for *Salmonella* species.

Water and milk samples were artificially contaminated with *Salmonella* and then subjected to antisense PNA treatment. Elongated cells were observed (Figures 5 and 6) with a turnaround sample analysis time of 16 h. The detection of *Salmonella* in artificially contaminated milk proved difficult at first and was associated with the presence of PNA inhibitors especially high salts (Mg^{2+} salts), fats and proteins. A sample clean up preparation step to remove the inhibitors (desalting) was incorporated into this procedure to improve PNA bioavailability and detection efficiency. Following this stage, the results were comparable to other developed diagnostic methods, which detected *Salmonella* in several artificially contaminated complex samples within 24 h [21]; 12–20 h [4]; 20–24 h [6, 10, 29, 30]. The detection of monoculture strains of *Salmonella* took a shorter time usually after 6 hour incubation period, which is similar to the detection turnaround for other rapid methods like PCR or FISH, and with the potential to advance the diagnosis of *Salmonella* and other bacteria.

This study is the first to assess the use of antisense technology for simultaneous phenotypic and genotypic bacterial diagnostics. The findings indicated a potential for using antisense gene silencing technology for detection of specific *Salmonella* species through suppressing cell division (*ftsZ* gene) and an alteration in *Salmonella* morphology. Antisense PNA strategy could be a potential rapid tool for bacterial detection in a

single reaction and could be useful as a method for viable bacteria investigations in foods and environmental samples. Hence, this work is considered a preliminary proof of concept, and further work will be to estimate the specificity and sensitivity of the method, as well as the potential for practical application in real samples. There will be the need to validate further the specificity and sensitivity of methods by including a wider range of *Salmonella* strains and non- *Salmonella* related bacteria.

Declarations

Author contribution statement

Oluwawemimo O. Adebowale: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Shan Goh: Conceived and designed the experiments; Wrote the paper.

Liam Good: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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