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ORIGINAL ARTICLE

Detection and differential identification of typhoidal *Salmonella* using bacteriophages and resazurin

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Abstract

This study was aimed at developing an easy to use and inexpensive biosensor for the detection of typhoidal *Salmonella*. The technique was designed to be used without expensive instrumentation if necessary. Bacteriophages specifically infecting three typhoidal *Salmonella* serovars were isolated and purified. Log-phase cultures were mixed with a high titre of a single phage (10^9 PFUs) in separate wells of a microtitre plate and incubated at room temperature (30 °C) for 1 h. After incubation, resazurin was added and the plates were incubated further for 1 h. Absorbance at 570 nm of each test well was measured using a commercial microplate reader and compared with that of the control well. A significant difference ($p < 0.05$) between the absorbance of test and control wells indicated the presence of target bacteria. With visual inspection, a delay in colour change from blue to pink was considered a positive result. The system could detect 5×10^4 CFUs in 120 min without pre-enrichment and 10 CFUs with a pre-enrichment of 6 h.

Keywords *Salmonella* · Biosensor · Bacteriophage · Viability · Rapid detection

Introduction

Salmonella is a zero-tolerance enteric pathogen that continues to be a major health problem throughout the world, with the highest number of cases reported in the Indian subcontinent (Wain et al. 2015). Recent estimates state that approximately 21 million typhoid-related cases occur worldwide annually, resulting in around 222 000 deaths (WHO 2014). In 2015 alone, 12.5 million new cases of typhoid fever were reported (GBD 2015). The Widal agglutination test has been the most widely used serological test for the diagnosis of typhoid fever for over a 100 years. Yet, it inherently shows only moderate specificity and selectivity (Sherwal et al. 2004) and has no diagnostic significance in patients in endemic regions (Olopoenia and King 2000). *Salmonella* detection by cultural and biochemical methods is generally considered reliable. However, these methods take days to detect and identify the pathogen to the species and serovar level. Moreover, use of such methods requires a skilled workforce which may not always be available. Additionally, a laboratory setup consisting of sophisticated instruments such as surface plasmon resonance (SPR) and polymerase chain reaction (PCR) machines may prove to be expensive, thus limiting the reach of such rapid methods to regions with financial difficulties. Efforts have been made to reduce the cost of such expensive technology by development of cheaper alternatives. However, they have met with limited success, owing to a lack of demand from users who prefer the advantages of sophisticated instruments (Turner 2013). It is therefore necessary to develop alternate, easy-to-use and scalable rapid methods for identification of *Salmonella,* which involve minimum use of expensive instruments.

Bacteriophages are obligate intracellular parasites of bacteria, which rely on their hosts for propagation. They generally infect their bacterial hosts with high specificity, making them ideal candidates for target pathogen detection. Bacteriophages are inexpensive and easy to produce in high titres, and maintain their titres for long durations under refrigeration. Apart from offering selectivity,

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bacteriophages are capable of 'auto-dosing', in that they increase in number in the presence of suitable hosts (Loc-Carrillo and Abedon 2011). This is especially useful for a biosensor, as the biosensing element does not get saturated and regenerates itself. Resazurin was chosen as the redox indicator for the viability assay, as it is relatively less toxic, inexpensive and more sensitive than the tetrazolium assays (Riss et al. 2013).

The sensing system developed in this study works on the principle of viability of target bacterial cells in the presence of an infecting phage (Fig. 1). Viable cells reduce resazurin, a blue dye, to resorufin, which is pink. However, in the presence of a suitable phage, target bacterial cells in the sample are killed, and the resazurin retains its blue colour. Thus, an absence of or a delay in a colour change from blue to pink can be considered as a positive result. This study combines the specificity of the bacteriophages with a cell viability assay based on resazurin, to develop an optical–visual biosensor for differential detection and identification of typhoidal *Salmonella*.

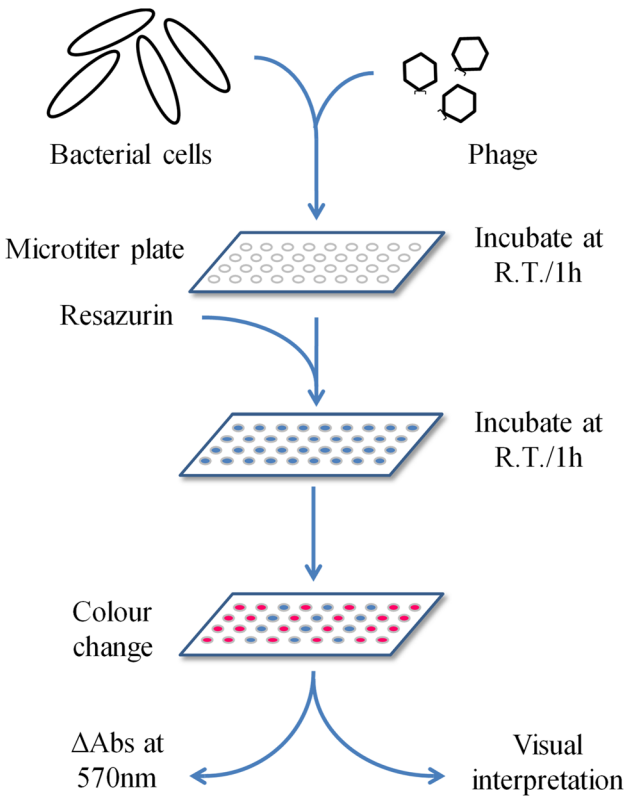

Fig. 1 Process flow for detection of typhoidal *Salmonella* serovars using the BRV assay

Methods

Bacterial cultures

Bacterial cultures of *Salmonella enterica* serovars Typhi, Paratyphi A and Paratyphi B used for the isolation of bacteriophages were obtained from a local hospital. The cultures were designated as *S. typhi* H-1, *S. paratyphi* A H-1 and *S. paratyphi B* H-1. Various laboratory isolates of multiple bacterial strains were used for host range studies according to methods previously described (Kutter 2009). For real sample analysis, cultures of the three typhoidal *Salmonella enterica* serovars were obtained from a local hospital and a pathological laboratory. The genus and species of the cultures were confirmed using biochemical and serological methods at the source. Log-phase cultures grown in Luria Broth (LB) (HiMedia Laboratories, India) to an OD_{600} of 1.0 were used for all the assays, and if necessary diluted with Luria broth unless stated otherwise. The cultures were maintained on Luria Agar (LA) (HiMedia Laboratories, India) plates and 50% glycerol stocks were made for storage at − 20 °C.

Resazurin suspension

Resazurin suspension was prepared according to the methods adapted from a previously described protocol. (Riss et al. 2013). Briefly, analytical-grade resazurin (HiMedia Laboratories, India) was dissolved in PBS (pH 7.4) to 0.034 mg ml^{-1} to prepare a stock. The solution was then filtered through a 0.22 µ filter and stored at − 20 °C in dark. This solution was diluted four times (0.0085 mg ml^{-1}) and used for further analyses.

Isolation, enrichment and storage of bacteriophages

Salmonella phage vB_Sen-UP2 and Salmonella phage vB_Sen-UP5 were isolated from sewage samples from Pune, India, while Salmonella phage vB_Sen-DN1 was isolated from a sewage sample from Dombivli, India. Isolation and enrichment were performed using the three *Salmonella enterica* serovars Typhi, Paratyphi A and Paratyphi B as hosts according to methods previously described (Van Twest and Kropinski 2009). Bacteriophages having selective infectivity against the individual serovars were selected for further studies. The bacteriophages were named according to the rules previously described (Adriaenssens and Brister 2017).

Bacteriophage suspensions of high titre (~ 10^{10} Plaque Forming Units (PFUs) ml^{-1}) were prepared using the technique described (Swanstrom and Adams 1951). The

suspensions were centrifuged at 6000 × *g* for 20 min to sediment debris, followed by filter sterilization through a sterile 0.45 µm filter and then stored at 4 °C for further use.

Characterization of bacteriophages

Transmission electron microscopy (TEM)

Transmission electron microscopy studies of the phages were carried out according to methods previously described (Ackermann 2009a). Briefly, 1 ml of each phage suspension was sedimented at 25,000 × *g* for 60 min. The supernatant was discarded and the sediment was washed twice with 1 ml 0.1 mol l^{-1} ammonium acetate (pH 7.0). The final sediment was resuspended in ammonium acetate. This suspension was then negatively stained on a carbon-coated copper grid with two % phosphotungstic acid and examined in a PHILIPS CM 200 Electron Microscope.

Pulsed field gel electrophoresis (PFGE)

Phage genome size was determined by PFGE. Agarose plugs containing bacteriophages were prepared using methods described (Lingohr et al. 2009). The agarose plugs, along with a thin slice of the Lambda Phage DNA Ladder (New England Biolabs, USA), were carefully inserted into individual wells in 1% SeaKem Gold Agarose gel (Lonza, USA). The wells were sealed using agarose. PFGE was performed at six V/cm with incremental pulses of 2.2–54.2 s for 24 h, in a Gene Navigator PFGE system (Amersham Biosciences, UK). Post-electrophoresis, the gel was removed, stained with ethidium bromide and visualized using a BXT 20 M UVIvue transilluminator (UVItec, UK).

Phage host range studies

Multiple strains of *Salmonella* Typhi, Paratyphi A, Paratyphi B, non-typhoidal *Salmonella* and other bacteria known to cause blood-stream infections were screened for susceptibility to the phages. Suspensions of bacterial cultures (300 µl) were mixed with 0.7% molten soft agar and overlaid on LA plates. After the soft agar had solidified, 5 µl of each bacteriophage lysate was spot inoculated onto the plates and incubated at 37 °C for 5–6 h.

Optimization of bacteriophage-resazurin viability (BRV) assay parameters

A volume of 10 µl of bacteriophage suspension (10^{10} PFUs ml^{-1}) was mixed with 90 µl of bacterial suspension (1.0 OD_{600}) in a microtitre plate well and incubated at 30 °C for 1 h. Similarly, 10 µl of phosphate-buffered saline (PBS) was added to 90 µl of bacterial suspension in another well and used as a negative control. After incubation, 10 µl of resazurin (0.15 mmol l^{-1}) solution was added to both the wells and the plate was incubated at 30 °C for 1 h. Absorbance at 570 nm was measured in a Biotek Synergy H1 microplate reader.

To test the effect of incubation temperature on the performance of the assay, another set of experiments was performed with the same setup, where two separate microtitre plates were incubated at 25 °C and at room temperature (R.T.) (30 °C).

To study the effect of diluents, two separate sets of assays were set up as mentioned above, where one set utilized LB as a diluent, while the second set used PBS.

To optimize incubation time, the above assay was carried out in multiple sets, where bacterial cultures were incubated with bacteriophages over a range of incubation times, from 15 to 60 min, at intervals of 15 min.

The minimum absorbance of resorufin at 570 nm required to be able to identify a colour change from blue to pink with naked eyes was determined. Briefly, viability assays involving a positive and negative control were set up in triplicate, and incubated at R.T. for 1 h. Resazurin was added to the test and control wells. The absorbance of the test wells was determined immediately after a change in colour from blue to pink could be observed.

To determine minimum pre-enrichment time at static conditions, an *S. typhi* H1 culture was diluted to 1-$\log_{10}$ CFUs $well^{-1}$ and incubated at 30 °C. Absorbance readings at 600 nm were determined at intervals of 15 min, till the OD_{600} reached 0.1.

To determine the limit of detection, a BRV assay involving twofold dilutions of *S. typhi* H-1 from 4-$\log_{10}$ to 8-$\log_{10}$ CFUs was set up and absorbance at 570 nm was measured at the end of the assay.

Interference from non-target bacterial cells

To study the effect of the presence of non-target bacterial cells in a sample, *Salmonella* Typhi and *Salmonella* Paratyphi A cultures were mixed in various proportions and tested against a single phage—DN1, which infected *Salmonella* Typhi, but not *Salmonella* Paratyphi A.

Reproducibility

Salmonella Typhi (H-1) culture (7 ± 0.16 $\log_{10}$ CFUs) was tested consecutively against DN-1. The difference in absorbance between the test and control was determined for each run, and relative standard deviation (RSD) was calculated to study the reproducibility of the biosensor.

Field sample analysis

In a blind test, the proposed biosensor was used to identify and differentiate between ten clinical samples of three *Salmonella enterica* serovars—Typhi, Paratyphi A and Paratyphi B, obtained from the pathology laboratory. Bacterial suspensions were incubated separately with the three different phages in microtitre plate wells for 1 h, followed by determination of absorbance at 570 nm. Two separate set of wells—one containing bacterial cultures alone, and the other containing a known culture with its infecting phage, were used as a negative and positive control, respectively.

Statistical analysis

All the experiments were performed in triplicate, and statistical analyses of absorbance readings were performed with Student's *T* test (95% confidence interval) to test for significance. Relative standard deviation (R.S.D.) was determined to study the reproducibility of the biosensor.

Results

Characterization of bacteriophages

TEM of the phages DN1 and UP5 revealed virions with icosahedral heads, 60 nm in diameter and short, non-contractile tails (Online Resource 1). The genomes of these phages were approximately 37.7 kb and 41.7 kb in size, respectively (Online Resource 2). TEM images of UP2 showed phage virions with icosahedral heads of 60 nm size and long, non-contractile tails (Online Resource 1). PFGE demonstrated that the genome of UP2 was 50 kb in size (Online Resource 2). This data, when compared with the available literature, concluded that DN1 and UP5 were Podoviridae phages, while UP2 belonged to the family Siphoviridae (Ackermann 2009b; Le Mercier 2010). These genome sizes are consistent with those reported earlier for Podoviridae and Siphoviridae phages infecting *Salmonella* (De Lappe et al. 2009; Karpe et al. 2016).

Table 1 Lytic activity of phages against various bacterial hosts

Bacterial host	DN1	UP2	UP5
Salmonella Typhi[a]	+	−	−
Salmonella Paratyphi A[a]	−	+	−
Salmonella Paratyphi B[a]	−	−	+
Pseudomonas aeruginosa (NCIM 2036)	−	−	−
Proteus mirabilis[b]	−	−	−
Klebsiella aerogenes[b]	−	−	−
Staphylococcus aureus[b]	−	−	−
Staphylococcus aureus[b]	−	−	−
Escherichia coli (ATCC 8739)	−	−	−
Escherichia coli (ATCC 35218)	−	−	−
Shigella dysenteriae[b]	−	−	−
Shigella dysenteriae[b]	−	−	−
Salmonella typhimurium (MTCC) 98	−	+	−
Salmonella typhimurium[b]	−	+	−

[a]Acquired from a local hospital

[b]Laboratory isolate

+:Lysis

−: No lysis

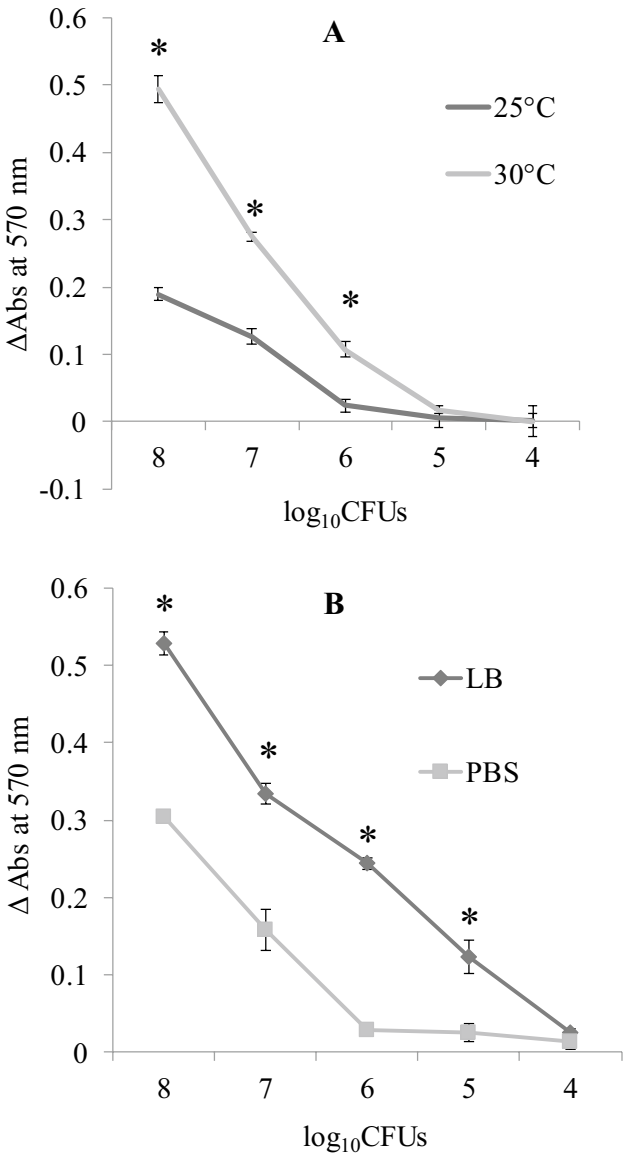

Fig. 2 **a** ΔAbs for assays conducted at 25 °C and at 30 °C. **b** ΔAbs for assays utilizing LB and PBS as diluents. Statistically significant ($p < 0.05$) differences between ΔAbs values of assays utilizing LB and PBS are marked with an asterisk (*)

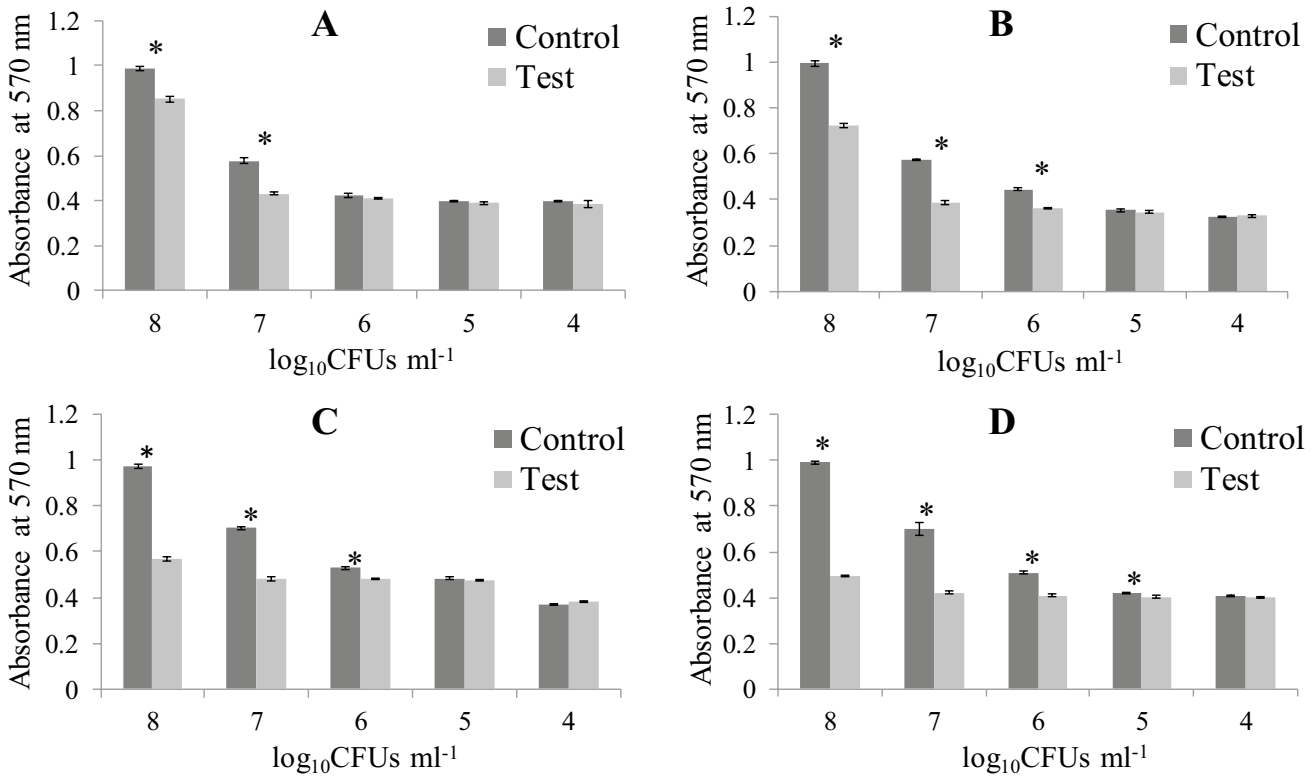

Fig. 3 Absorbances of test and control wells at 15 min (**a**), 30 min (**b**), 45 min (**c**) and 60 min (**d**). Significant differences in absorbance of test and control wells are marked with an asterisk ($p < 0.05$, $n = 3$). Difference in absorbance at 3-$\log_{10}$ CFUs and below is not shown

None of the phages isolated against a single typhoidal *Salmonella* serovar was able to infect the other typhoidal serovars, which shows high specificity of the recognition elements for their target, up to serovar level. Host range studies conducted against other bacterial genera demonstrated that DN1 and UP5 had a narrow host range, and specifically targeted the *Salmonella* serovars that they were isolated against (Table 1). Incidentally, UP2 showed lytic activity against both the *Salmonella typhimurium* isolates used in the study. This is potentially advantageous, as certain Typhimurium strains of invasive non-Typhi *Salmonella* (NTS) have been implicated in causing blood-stream infections (Morpeth et al. 2009; Feasey et al. 2012).

Optimization of BRV assay parameters

The studies to determine the effect of incubation temperature on the assay indicated that the optimum incubation temperature was 30 °C. The difference between the absorbances of test and control wells (ΔAbs) at 30 °C (0.018 ± 0.005) was found to be higher than the of ΔAbs at 25 °C (Fig. 2a). At a cell density of 5-$\log_{10}$ CFUs, the ΔAbs at 25 °C (0.005 ± 0.013) was no longer statistically significant ($p > 0.05$). This reduction in sensitivity may be attributed to a reduced host metabolic rate at sub-optimum temperature and reduced phage propagation and host lysis. This conforms with earlier reports, which suggest that low temperature may slow down overall host metabolism, decreasing host growth and phage propagation (Tey et al. 2009).

The ΔAbs increased with an increase in the incubation time (Fig. 3). A longer incubation time allowed for multiple phage infection cycles, leading to a higher loss of viability of the host, thus increasing the ΔAbs. An incubation time of 60 min was sufficient to get statistically significant ($p < 0.05$) ΔAbs for wells that started with 5-$\log_{10}$ CFUs. The wells with cell density lower than 5-$\log_{10}$ could not produce a detectable colour change, giving insignificant ΔAbs values ($p > 0.05$). Thus, the response time of the biosensor would be 2 h, including the incubation of target cells with phages for 1 h, and the incubation post the addition of resazurin to the wells.

Assays performed using a rich medium (LB) and PBS as diluents in separate sets (Fig. 2b) demonstrated that the biosensor performed better with LB, showing higher ΔAbs (0.124 ± 0.021) as compared to that obtained with PBS (0.026 ± 0.011). In the presence of LB, the viable host cells were allowed to grow and increase in number, thus increasing the ΔAbs, which was desirable. However, the same was not observed with PBS as the diluent, and

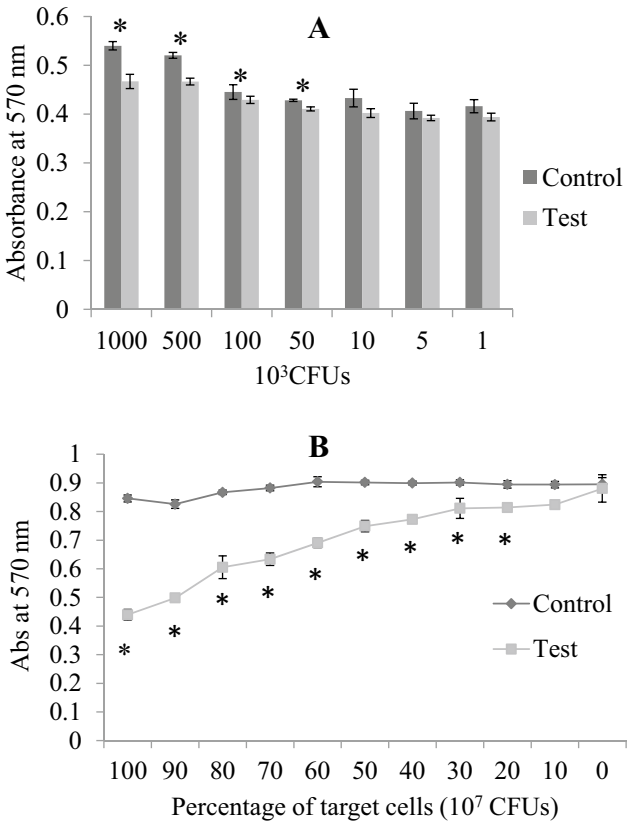

Fig. 4 **a** Limit of detection of the BRV assay towards target cells. Data points with statistically significant ($p < 0.05$) ΔAbs values are marked with an asterisk (*). **b** Interference from non-target bacterial cells at various proportions of target and non-target cells. Significantly different absorbance values of test wells as compared to control wells are marked with an asterisk ($p < 0.05$, $n = 3$)

hence the ΔAbs values were higher with LB than those obtained with PBS.

A minimum absorbance of 0.53 ± 0.0085 at 570 nm could be detected visually. The ability to visually detect the presence of a target pathogen in the sample mitigates the requirement of a transducer (Online Resource 3).

With a minimum static enrichment of 6 h, an OD_{600} of 0.1 was achieved from as few as 1-$\log_{10}$ CFUs well^{-1}. Thus, with pre-enrichment, the biosensor can detect as low as 10 CFUs well^{-1}. Without the pre-enrichment, the biosensor was able to detect as low as 5×10^4 CFUs well^{-1} (Fig. 4a). Results of the optimization studies have been summarized in Table 2.

Interference from non-target bacterial cells

Interference studies indicated that a minimum of 20% of target cells in a mixture of target and non-target bacterial cells were sufficient for successful detection of the target by the biosensor. Thus, in a real scenario, the biosensor will not be affected by the presence of up to 80% of interfering non-target bacterial cells in a sample. The ΔAbs decreased with decreasing percentage of target cells (Fig. 4b). At 10% of target cells this difference was not statistically significant ($p > 0.05$).

Reproducibility

The biosensor was tested multiple times at a cell concentration of 7 ± 0.16 $\log_{10}$ CFUs of *Salmonella* Typhi (H-1) and showed good reproducibility (Fig. 5). The RSD value of 2.22% ($n = 10$) so obtained was lower than many other *Salmonella* detection platforms (Wong et al. 2002; Su and Li 2005; Zhang et al. 2012).

Field sample analysis

The biosensor was able to correctly identify all the typhoidal *Salmonella* cultures from the clinical samples obtained from the pathological laboratory. The results obtained using the BRV assay matched with the serological, biochemical and PCR tests conducted by the laboratory (Online Resources 4, 5 and 6).

Discussion

The biosensor has significant implications with respect to the detection of bacterial pathogens in developing countries. This detection system bypasses the cultural methods of pathogen identification, and brings down the time of identification from days to hours. The BRV assay had a working range of 20% and above in terms of percent target cells in the sample, which indicates that this biosensor would not be affected by the presence of non-target cells in this range. The system has no strict requirement of a transducer, as the result can be interpreted visually. This is especially important with respect to making rapid detection systems affordable in low budget scenaros. Although the BRV assay can be far cheaper

Table 2 Optimized parameters for the BRV assay

Temperature (°C)	Total Incubation time (h)	Diluent	Minimum static pre-enrichment time (h)	Minimum Abs at 570 nm for visual detection	Limit of detection (CFUs well^{-1})	
					With pre-enrichment	Without pre-enrichment
30	2	PBS (pH 7.4)	6	0.53 ± 0.0085	10	5×10^4

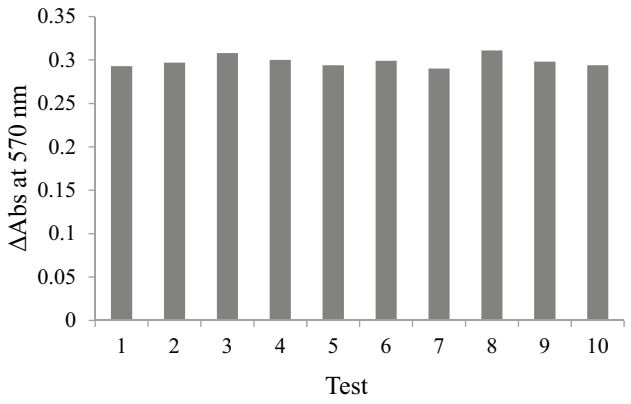

Fig. 5 Difference in absorbance between test and control wells for ten consecutive runs

than PCR-based assays that require thermocyclers, certain isothermal amplification systems such as Loop-mediated Isothermal Amplification (LAMP), cross-priming amplification (CPA) exists that does not require expensive equipment to work. A LAMP method was described that can detect 100 CFUs of *Salmonella* per reaction in approximately 60 min (Zhao et al. 2010). Similarly, a cross-priming amplification (CPA)-based method was reported for the detection of *Listeria monocytogenes* within 90 min with a limit of detection of 2.5 pg DNA per reaction, and the results could be determined by visual inspection (Wang et al. 2014). The ability to visually inspect the result makes detection of targets even easier. Moreover, most of the optical detection platforms today have been integrated with smartphones using the phone camera as a detector (Roda et al. 2016). This means that the BRV assay may be integrated with a smartphone or a similar device instead of an expensive transducer. The biosensor works very well at room temperature and therefore does not require an incubator for maintaining temperature. This can greatly reduce operational cost and maintenance. The sensor can be expected to have high stability and long shelf life, as the components are stored separately and can be maintained under refrigerated conditions for long periods of time (Ackermann et al. 2004; Riss et al. 2013). Additionally, the BRV assay is conducted in a microtitre plate and is therefore scalable. Lastly, the phage(s) used as biorecognition elements can be changed so as to target other bacterial pathogens, rendering this biosensor highly amenable to modification. All of these advantages make this biosensor a rapid, inexpensive, scalable, and easy to use alternative to conventional methods of pathogen detection.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflicts of interest to disclose.

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