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REVIEW

Advances in detection methods for viable *Salmonella* spp.: current applications and challenges

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Abstract

Salmonella is a common intestinal pathogen that can cause food poisoning and intestinal disease. The high prevalence of *Salmonella* necessitates efficient and sensitive methods for its identification, detection, and monitoring, especially of viable *Salmonella*. Conventional culture methods need to be more laborious and time-consuming. And they are relatively limited in their ability to detect *Salmonella* in the viable but non-culturable status if present in the sample to be tested. As a result, there is an increasing need for rapid and accurate techniques to detect viable *Salmonella* spp. This paper reviewed the status and progress of various methods reported in recent years that can be used to detect viable *Salmonella*, such as culture-based methods, molecular methods targeting RNAs and DNAs, phage-based methods, biosensors, and some techniques that have the potential for future application. This review can provide researchers with a reference for additional method options and help facilitate the development of rapid and accurate assays. In the future, viable *Salmonella* detection approaches will become more stable, sensitive, and fast and are expected to play a more significant role in food safety and public health.

Keywords Viable *Salmonella* · Detection methods · Culture-based methods · Nucleic acid detection · Phage-based methods · Biosensors

Introduction

Salmonella is a Gram-negative, non-spore-forming, rod-shaped bacterium belonging to the Enterobacteriaceae family and is the leading cause of foodborne illness worldwide [1]. The genome of *Salmonella* ranges between 4460 and 4857 kb and varies between serotypes. *Salmonella* is a lactose fermentor (some subspecies) and bisulfite producer and is oxidase-negative and catalase-positive [2, 3]. *Salmonella* hydrolyses urea, using citrate and decarboxylated lysine as its sole carbon source [4].

Salmonella species harbor in the intestines of humans and farm animals. Reptiles and insects are also hosts for *Salmonella*. In addition, eggs, poultry, pork, beef, dairy products, nuts, vegetables, and water are sources of *Salmonella*. The risk of infection is high in low- and middle-income countries or societies, with more than 100 infections per million people per year [3, 5]. According to the World Health Organization (WHO), *Salmonella* ranks first among 22 critical foodborne pathogens [6]. And *Salmonella* is the second leading causative agent of foodborne pathogens in the EU and the USA, preceded by *Campylobacter* spp. and norovirus [7, 8]. *Salmonella* causes an estimated 10,000 cases of foodborne illness in the United States annually, accounting for 28% of hospitalizations and 35% of deaths attributed to known foodborne bacterial pathogens [9, 10]. In Asia, bloodstream infections caused by *Salmonella enterica* account for 30% of

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all community-acquired infections, while in Africa, 29.1% of community-acquired bloodstream infections are caused by the same *Salmonella* species [11, 12]. The high prevalence of *Salmonella* necessitates efficient and sensitive methods for identification, detection, and monitoring, especially for viable *Salmonella* spp.

Growth and division are the main reference indicators for identifying bacterial viability, usually through the ability of bacteria to multiply in nutrient media. However, it has been found that many bacterial species exist in a viable but non-culturable (VBNC) state [13–15]. VBNC bacteria are characterized by a loss of culturability on conventional media. Therefore, they cannot be tested by traditional culture methods, leading to an underestimation of the total number of viable bacteria in the study population. However, pathogenic bacteria in the VBNC state have intact cell membranes and maintain a membrane potential, unlike dead bacteria cells whose membranes have disintegrated. Besides, VBNC bacteria retain virulence and can recover and cause infection under favorable conditions. Studies have shown that *Salmonella* induces a VBNC state when exposed to nutrition starvation, salt stress, low-level acidity, and low temperatures [16]. Therefore, accurate detection of viable bacteria, especially pathogens in the VBNC state, is essential for scientific prevention and control of outbreaks.

Culture-based counting methods have been the "gold standard" for quantifying viable bacteria. In addition to being used to measure the number of viable bacteria in food, it is also commonly used to test water quality [17]. However, it has been found that not all viable bacteria can be cultured. On the one hand, artificial culture conditions are challenging to meet the growth requirements of many bacteria, resulting in the inability of viable bacteria to grow on the medium. On the other hand, bacteria exist in the VBNC state and when cultured under conventional conditions, they do not grow and multiply, but remain metabolically active. In addition, selective enrichment cultures, biochemical analysis, serological identification, molecular identification, and mass spectrometry are often necessary to confirm specific information about the isolate [18]. Moreover, the non-uniform distribution and low abundance of pathogens in food samples, the heterogeneity of food matrices, and the presence of indigenous bacteria that may interfere with the isolation of specific pathogens can all affect the accuracy of culture results [19]. As a result, culture-based methods often struggle to meet diverse and realistic needs. To overcome the limitations of culture-based methods, researchers have proposed a variety of more rapid, alternative, culture-independent methods to improve the detection of viable bacteria.

This paper classified and summarized the various published methods for the rapid detection of viable *Salmonella*, including culture-based methods, molecular methods targeting RNAs and DNAs, phage-based methods, biosensors, and other innovative methods (Scheme 1). And we initially discussed the advantages and possible limitations of these methods to provide a methodological reference and theoretical basis for research and applications related to the detection of viable *Salmonella* spp.

Culture-based methods

Membrane integrity-based staining assays

The integrity of the cell membrane is a recognized characteristic of a viable cell, as cells with damaged membranes are near death or already dead [20]. There are several ways to test cells' membrane integrity and viability. Polar stains cannot penetrate surviving cells with intact membranes but can penetrate dead cells with damaged membranes [21, 22].

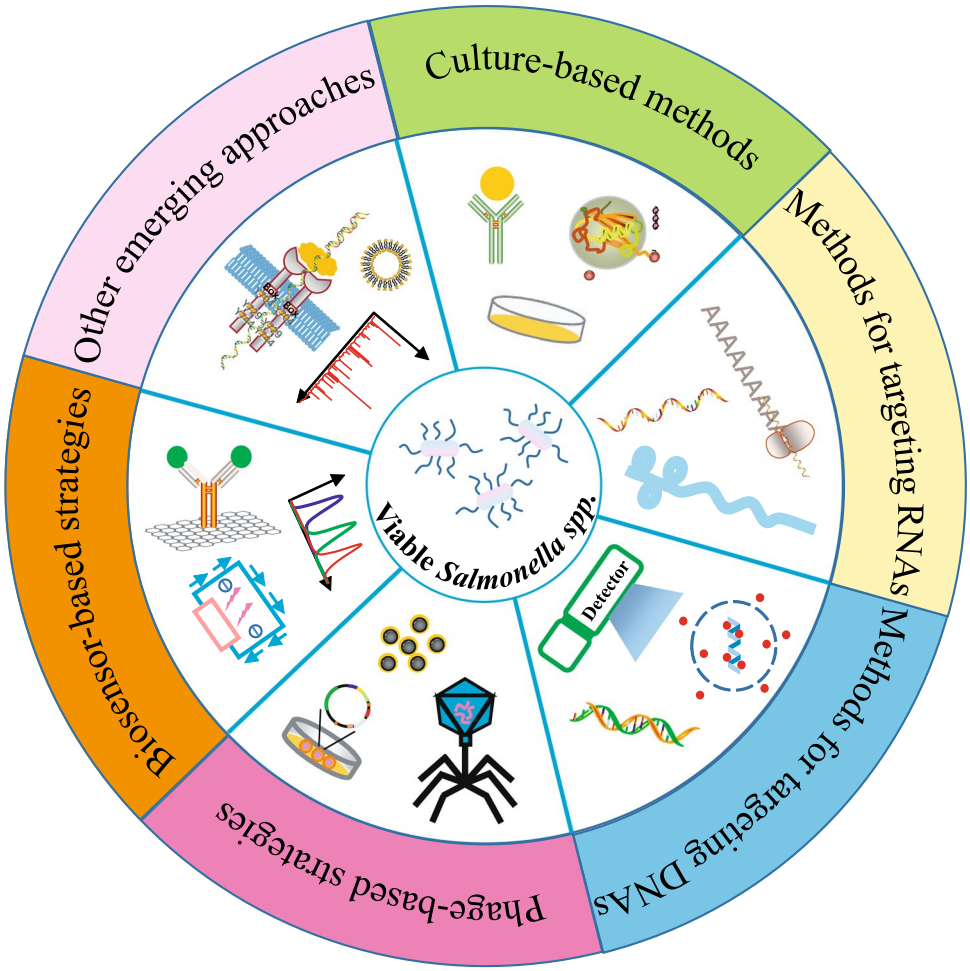
The use of staining has shown obvious advantages in the detection of VBNC bacteria. Staining is a method that directly indicates the ratio of viable to dead cells and allows the membrane integrity of VBNC bacteria to be detected. Methods based on dual staining reagents, such as the LIVE/DEAD BacLight™ bacterial viability system, can be used to detect VBNC *Salmonella* [10]. It can also be used to observe the dynamics of dead and living cells over time. However, it is worth noting that the structure of cell membranes and their composition varies considerably in different bacteria, so it remains challenging to use membrane integrity as a biomarker to analyze viability in mixed cultures or environmental samples [23].

Duffy et al. detected viable pathogens by extracting overnight cultured *Salmonella* cells onto a polycarbonate membrane and then using anti-*Salmonella* monoclonal antibodies linked to an antibody-conjugated fluorescent stain (Texas Red) and a vitality stain (Sytox Green) [24]. This direct staining method could detect viable *Salmonella* in fresh and processed meat.

To circumvent the limitations of reactive dye extrusion or cell dormancy on enzyme activity, Caron et al. developed a rapid triple fluorochrome staining procedure that actively excluded ethidium bromide (EB) (metabolically active cells), absorbed EB but excluded peroxisomes and allowed uptake of two dyes (depolarization) [25]. Permeabilized cells were identified by uptake of propidium iodide (PI).

In addition, flow cytometry makes it possible to distinguish between live and dead bacteria based on fluorescence results [26, 27]. Flow cytometry (FCM) is a technique that can be used to rapidly determine a target's optical and fluorescent properties. It can be used to perform colony counts and the analysis is not dependent on microbial growth, allowing the detection of bacteria that cannot be grown on agar plates (e.g. VBNC bacteria). Besides, the addition of counting beads to the LIVE/DEAD BacLight™ Bacterial

Scheme 1 Current methods for the detection of viable *Salmonella* spp.



Viability Kit allows conversion of FCM data to the number of cells per unit volume (Fig. 1) [28, 29]. Flow cytometric analysis has recently been increasingly used to detect live bacteria. Wang et al. efficiently detected single viable *Salmonella* cells within 7 h using FCM by double staining with fluorescein isothiocyanate (FITC)-labeled anti-*Salmonella* antibodies and PI dye [30]. Khan et al. reported an optimized staining protocol and method for FCM that allowed the enumeration of VBNC bacterial cells within 70 min [31]. Experiments were performed using FCM to quantify VBNC *Salmonella* Typhimurium cells after staining with different fluorescent probes (e.g., SYTO 9 and PI). The FCM data

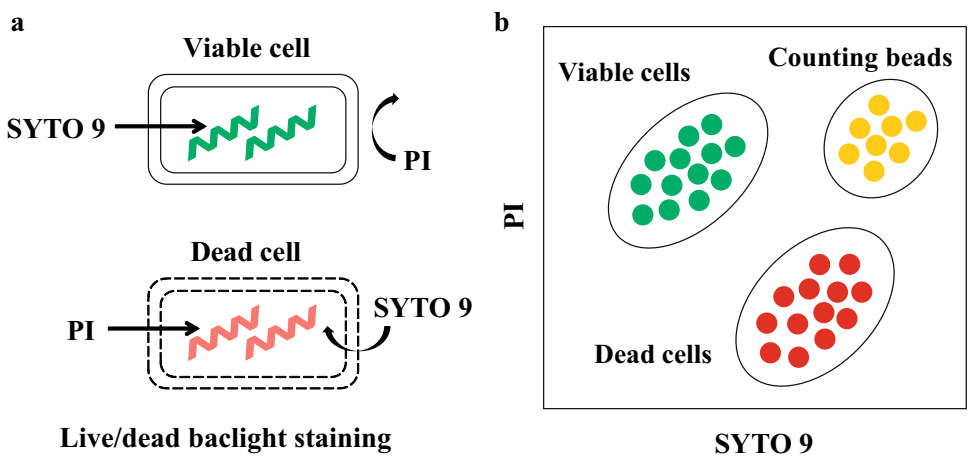


Fig. 1 Schematic diagram of live/dead backlight staining and its application in combination with FCM. **a** Staining of viable and dead bacteria using the LIVE/DEAD BacLight™ Bacterial Viability Kit show green and red, respectively. [28] **b** Dot plot of SYTO 9 fluorescence versus PI fluorescence. The gates with green, red and yellow dots represent viable cells, dead cells and counting beads, respectively [29]

were compared with data from standard nutrient agar, and the results showed the value of the method for rapid and unbiased detection of dead versus live organisms.

It is important to note that dye-based assays are susceptible to pH, dye concentration, user handling, and background light, and that dyes can pose a health risk to the operator. In addition, fluorescence microscopic observation of counts is unsuitable for detecting large sample volumes. Staining can also be combined with flow cytometry techniques for quantitative detection, significantly increasing automation. However, flow cytometry is still relatively expensive, requires a large amount of dye, and is unsuitable for large-scale testing.

Esterase substances-based assays

The esterase substrate diffuses into the cell due to its neutral charge and is converted into fluorescent material by the intracellular esterase. Thus, surviving cells producing esterase can convert the suspension into a fluorescent emitting substance, whereas in dead cells, the rest remains unhydrolyzed and inactive [32]. This method demonstrates the enzyme's activity and the cell membrane's integrity. Calcein, fluorescein diacetate, and carboxyfluorescein diacetate are commercially available esterase substrates used in viability studies. Esterase substrates such as calcein AM are converted to fluorescent end products in the presence of intracellular esterases that hydrolyze acetoxymethyl esters [33]. Similarly, fluorescein diacetate and carboxyfluorescein diacetate are converted to fluorescein by the action of intracellular esterases and retained in the cell, where they are observed by flow cytometry [34]. Roth-Konforti team reported two new chemiluminescent probes (CLSP and CLLP) for the ultra-sensitive direct detection of viable bacteria [35]. The CLSP and CLLP probes consist of phenoxy dioxane luminophores masked by trigger groups that could be activated by specific bacterial enzymes to detect their corresponding bacteria with a limit of detection approximately 600 times lower than that of fluorescent probes. In addition, the method could detect at least 10 *Salmonella* spp. in 6 h. The assay allowed bacterial enrichment and detection in a single tube without the need for an additional sample preparation step. Besides, ATP is also an indicator of metabolically viable cells. Hunter et al. detected *Salmonella* Typhimurium in foods down to 10^4 CFU mL^{-1} using a combined ATP bioluminescence immunoassay, independent of the presence of non-target cells [36]. Pal et al. used antibody-functionalized Zn-MNCs to capture and magnetically isolate *Salmonella* in milk with a detection limit of 10 CFU mL^{-1} using an ATP luminometer [37].

It is worth mentioning that the bioluminescence method requires simple equipment and reagents, is a rapid method and can give a more precise range of viable bacteria in a short time (Fig. 2). However, it is still less sensitive and susceptible to external factors (e.g., non-bacterial ATP, dilution concentration, temperature, acidity, alkalinity, etc.).

Applications of biosensors

Interdigitated microelectrodes (IMEs) are impedance sensors for rapid detection of *Salmonella* Typhimurium in a selective medium and milk samples [39]. Yang et al. found that there was a linear relationship between the detection time of the onset of impedance change and the logarithm of the initial cell count of *S*. Typhimurium in the culture medium and milk samples. Based on this method, a minimum of 1 viable cell in the sample could be detected. In 2006, they selectively isolated *S*. Typhimurium from the samples using immunomagnetic beads coated with anti-*Salmonella* antibodies [40]. They found by analyzing the equivalent circuit of the IME system that the impedance

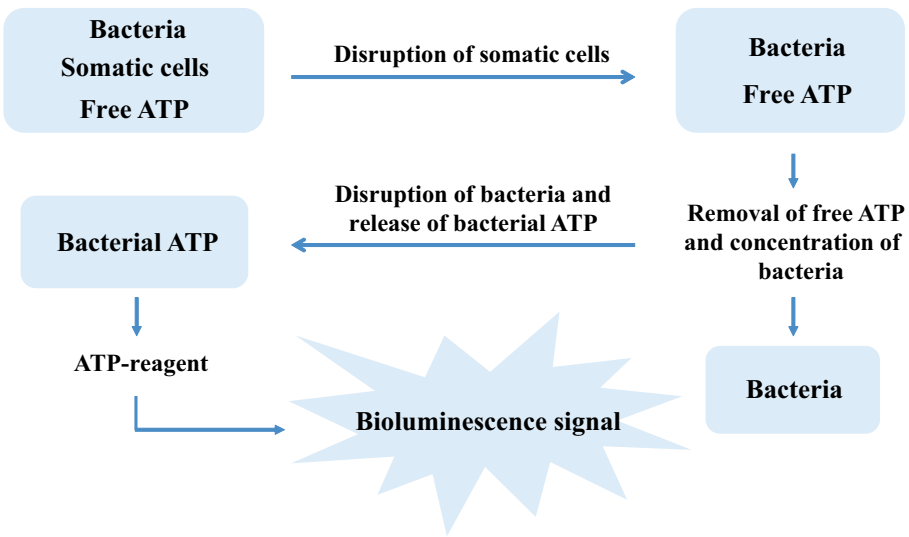


Fig. 2 A protocol for the bioluminescence determination of intracellular ATP in biological samples [38]

change in the BHI broth was a change in the bilayer capacitance due to the growth of *Salmonella*. It is worth noting that effective immunological separation of the target organism is required to obtain selectivity when using the non-selective medium.

Rapid and effective detection of foodborne pathogens is a societal priority as well as a scientific and technical challenge, as one viable cell needs to be detected in 25 g of food. Papadakis et al. pre-enriched one *Salmonella* cell in 25 mL of milk for 3 h and then used immunomagnetic beads to capture cells afterward, followed by DNA amplification using a loop-mediated isothermal amplification (LAMP) method and successful detection in combination with micro-/nanotechnology and acoustic waves [41].

Mantzila et al. constructed a faradic impedimetric immunosensor that can be used to detect *S*. Typhimurium in milk by cross-linking polyclonal anti-*Salmonella* on a gold electrode [42]. The sensor response increased with detection time due to the proliferation of viable *Salmonella* cells in the sample under test while virtually insensitive to dead cells.

Immunological assays

Surface plasmon resonance imaging is an optical method based on a biosensing chip capable of observing and quantifying the interactions of nano- and micron-sized objects near metal surfaces with both temporal and spatial resolution [43]. Based on surface plasmon resonance imaging of antibody arrays and bacterial growth during enrichment, the method established by Morlay et al. allowed the detection of 30 CFU of *Salmonella* cells in 25 g of powdered infant formula within 1 day [44].

Microfluidics allows millions of cells to be generated and evaluated in a matter of hours compared to traditional culture-based methods, which are limited by the growth rate, plate size, appropriate dilution, cell density, and the inability to culture certain environmental bacteria [45]. Harmon et al. used fluorescently-labeled anti-*S*. Typhimurium antibodies and relative fluorescence intensity, combined with in-droplet culture, to detect viable *Salmonella* within 5 h [46].

Other reported assays

Thiazole orange monoazide (TOMA), a new DNA dye that blocked DNA from dead bacteria, was introduced based on the metabolic activities of bacteria [47]. After 3 h of enrichment in pure cultures, recombinase-aided amplification (RAA) in combination with TOMA (TOMA-RAA) could detect 3.5×10^2 CFU mL^{-1} of viable *Salmonella* in samples.

In addition, viable *Salmonella* Typhimurium was detected by monitoring bacterial growth in selenite cystine broth supplemented with trimethylamine hydrochloride and mannitol using a three-electrode electrochemical impedance technique (1 Hz capacitance at a low frequency and 1 MHz resistance at a high frequency) [48].

The mammalian cell-based assays detect pathogen–host cell interactions and respond only to viable pathogens, but the short shelf-life of mammalian cells is a significant obstacle to their widespread use. Xu et al. extended the shelf-life of mammalian cells by using formalin and used them to capture viable pathogens, while specific detection was performed with antibodies [49]. The method allowed the detection of 1–10 CFU 25 g^{-1} of *S*. Enteritidis and *S*. Typhimurium in artificially infected materials within 12 h.

By combining culture with PCR, Löfström et al. developed a method for the simple and rapid detection of viable *Salmonella* with a detection limit of 1 CFU 25 g^{-1} of feed and a higher positive detection rate than the culture method [50]. Hice et al. used magnetic ionic liquid (MIL) solvent to extract and concentrate viable bacteria and recombinase polymerase amplification (RPA) to detect *Salmonella*-specific DNA [51]. The MIL-RPA method allowed the detection of viable *Salmonella* at concentrations as low as 10^3 CFU mL^{-1}.

Fujikawa et al. developed a method for estimating the number of viable *Salmonella* Enteritidis cells by qPCR with 5'-nuclease based on *invA* of microorganisms grown in samples during culture [52]. As *Salmonella* cells grew, the CT values decreased over time, producing a downward sigmoidal curve. The slope of the curve was constant at different initial cell concentrations. An increase in cell concentration leaded to a decrease in CT value, which was seen in terms of slope over time. Dead *Salmonella* cells caused a deviation in the CT curve. The method has also been validated for various foods and applied to the estimation of live cell counts of the target microorganisms [53].

It has been suggested that the integrity of the membrane may be a very conservative criterion for determining microbial viability [54]. Nocker et al. suggested the use of "activity-labile compounds" to selectively detect cells with metabolic and respiratory activity (while excluding inactive dead cells from detection) [55]. In addition to their potential usefulness for viability assessment, these new compounds could facilitate the selective amplification of nucleic acids in cells with relevant metabolic activity. Additionally, the redox reaction between quinone and viable microorganisms produces active oxygen species. Yamashoji et al. measured the rate of active oxygen production by luminol chemiluminescence and found that the luminescence intensity was proportional to the number of surviving cells [56]. This chemiluminescence assay is simple, rapid, and applicable.

In general, culture-based methods are based on the reproductive growth of viable bacteria. Although culture-based methods are relatively inexpensive and easy to use, they require at least 2–3 days to produce results. Waiting for culture results causes a delay in detection and lacks timeliness.

The culture method is even more helpless for bacteria in a VBNC state unless the bacteria can be successfully restored from that state to a culturable state. Many studies have demonstrated that bacteria can be recovered under appropriate conditions, and various recovery methods exist. However, there is no universal method to recover all VBNC-status bacteria.

Methods for targeting RNAs

rRNAs

Ribosomal RNAs (rRNAs) are a class of housekeeping genes with high copy numbers and short half-lives (Fig. 3). As rRNA molecules are only transcribed in metabolically active cells and are rapidly degraded after metabolism has ceased, direct analysis of rRNA molecules can reveal the diversity and, to some extent, the number of metabolically active organisms [57, 58]. Xue et al. used nucleic acid sequence-based amplification (NASBA) to amplify 16S rRNA directly and utilized Cas13a/crRNA to identify amplicons for sensitive (close to 1 CFU and 1% viable bacteria detectable) and rapid (within 2.5 h) detection of viable *Salmonella* [59].

Fluorescence in situ hybridization (FISH) has been a promising method in this regard. The ability of FISH to differentiate viable from non-viable cells depends on the rapid degradation of rRNA in non-viable cells. Taking advantage of the cellular delivery properties of polyhexamethylene biguanide, Adebowale et al. used it in combination with FISH to deliver oligomers targeting 23S rRNA and to rapidly detect viable *Salmonella* [61]. This strategy enabled a fixation-free protocol and hybridization in a single reaction. Similarly, the study by Rathnayaka et al. also confirmed that rRNA-based FISH could distinguish viable *Salmonella* from non-viable cells, especially for samples subjected to extreme heat [62].

Typically, the propidium monoazide or ethidium monoazide-based PCR techniques result in a 3.5 log reduction in dead bacteria compared to the associated deadly bacteria without treatment. However, more than this difference may be required to completely inhibit DNA amplification in high concentrations of dead Enterobacteriaceae ($> 10^6$ cells mL^{-1}) in PCR due to the potential for contamination in pasteurized milk. With this in mind, Soejima et al. treated milk samples with 23 µM PMA to completely inhibit long DNA amplification of 16S-23S rRNA and then used RT-qPCR to amplify the above targets, with a detection limit up to 2.5 logs CFU per PCR against viable *Salmonella* Enteritidis [63].

mRNAs

Messenger RNA (mRNA) is considered to be a better indicator of cell viability than DNA, as this molecule is only present in metabolically live cells [64]. Therefore, detecting RNA, especially the highly unstable mRNA, is usually a better indicator of the presence of living cells than detection of DNA. Also, mRNA is only produced by metabolically viable cells, making mRNA suitable for specifically detecting living microorganisms [65, 66]. Quantity of mRNA can be correlated to cell viability and cells' ability to grow, i.e., their culturability [67]. In addition, using RNA as a detection material has the advantage of greater sensitivity, as there are even hundreds of RNA copies corresponding to a single gene. If the copy number is high enough, rapid detection of *Salmonella* can be achieved, and enrichment may not be necessary. It has been shown that by using NASBA to amplify

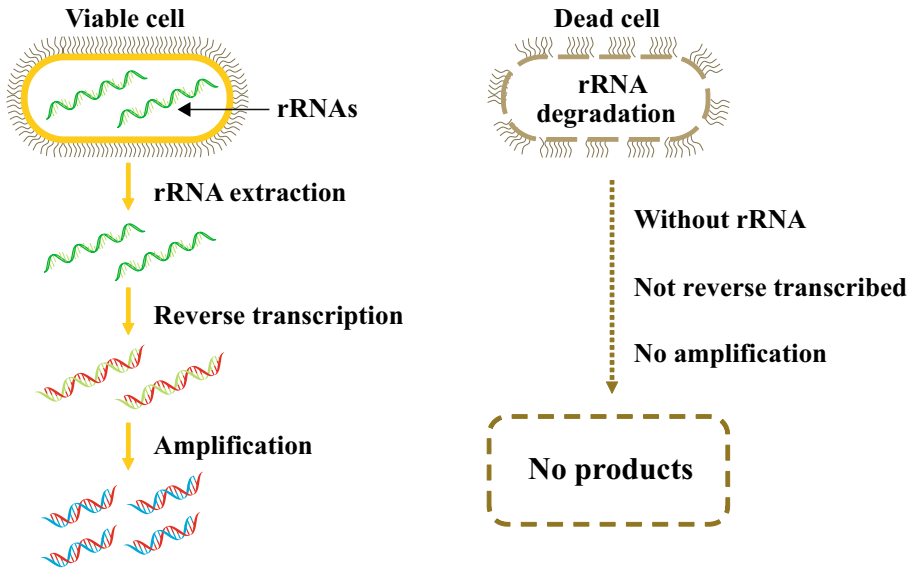


Fig. 3 Principle of viable bacteria detection based on rRNAs [60]

mRNA transcribed from the *dnaK* gene, there is a significant difference ($P > 0.01$) between the amplification signals of mRNA extracted from surviving and heat-dead cells of the same population [68]. Zhou et al. developed an RT-qPCR targeting *sigDE* mRNA for the specific detection of viable *Salmonella enterica*, which could detect *Salmonella* down to 1 CFU mL^{-1} from egg broth and milk after pre-enrichment [69].

To rapidly differentiate in vivo *Salmonella enterica*, Zhai et al. developed a NASBA method targeting *xcd* mRNA with high specificity and low detection limits [70]. González-Escalona et al. demonstrated that *invA* mRNA-based RT-qPCR could achieve results comparable to those of the FDA's Bacteriological Analytical Manual *Salmonella* culture method for viable *Salmonella* detection [71]. Miller et al. also demonstrated that *invA* mRNA can be used as a selectable biomarker for active *Salmonella*, with a detection limit of 10^4 CFU 25 g^{-1} *Salmonella* from enriched inoculated peppers by SYBR Green I-based RT-qPCR, which was consistent with a previous study [72, 73]. Also, VBNC *Salmonella* cells have been evidenced in manured soil using RT-PCR targeting *invA* mRNA [74]. In addition, antisense oligomers targeting *ftsZ* mRNA in combination with selective growth conditions could provide a detection strategy for viable *Salmonella* and a potential tool for bacterial detection in food and environmental samples [75].

Miao et al. evaluated RT-qPCR targeting tmRNA to detect live *Salmonella* spp. rapidly [76]. The results showed that the method could detect *Salmonella* spp. in fresh-cut vegetables with a sensitivity of 1 CFU mL^{-1}, regardless of 6 h of enrichment. In addition, combining RT-PCR targeting mRNA with electronic DNA microarrays allowed specific and sensitive detection of the survival of target *Salmonella* [77].

Cook believed that mRNA might be more advantageous than 16S rRNA for the specific detection of live cells [78]. However, the fallibility of mRNA as a suitable target for detecting live cells also makes the assay more challenging. Improper sample handling and storage or contamination of samples with RNA-degrading enzymes can lead to degradation. The expression levels of many mRNA species depend heavily on the cell's physiological state, which is often an unknown factor, complicating the quantification of the number of living cells. It is likely that when slow-growing or dormant cells are present in a sample, the RNA content of such cells is below the detection limit of RNA-PCR, while the cells remain largely viable or active [79]. In addition, it is essential to note that despite the static nature of mRNA, residual transcripts can show false positive signals in the presence of large numbers of dead bacteria [64]. For several reasons mentioned above, the application of detecting bacterial foodborne pathogens appears to be less common and is currently used mainly for inactivation studies or challenge tests [80, 81]. This may be because the method is too laborious or due to the rapid degradation of RNA in the test sample, which may also lead to false-negative results [82].

Other RNA targets

In 2004, Morin et al. designed primers based on *rfbE*, *fliC*, and *tyv* RNAs to establish a multiplex PCR (mPCR) that could simultaneously distinguish between the viable *E. coli* O157:H7, *Vibrio cholera* O1, and *Salmonella* Typhi [83]. The method could detect and identify as few as 30 cells of *E. coli* O157:H7 and *Salmonella* Typhi in clinical isolates. In addition, the RT-qPCR method established by Techathuvanan et al. targeting *invA* RNA could detect 1 CFU 25 mL^{-1} of viable *Salmonella* Enteritidis in egg samples within 16 h (Table 1) [84].

As transcription is one of the initial cellular responses to stimuli, using RNA as a molecular target for live bacteria is of biological significance. Furthermore, the average half-life of RNA is shorter than that of DNA, so RNA collected from environmental samples is most likely to represent living microorganisms. Since the average half-life of mRNA in active cells is a few minutes, the half-life of free molecules in the environment is even less. Methods focusing on mRNA allow specific microbial metabolic responses to be tracked quickly. Compared to mRNA, rRNA has a half-life of several days and is more abundant in cells. In addition, rRNA may allow for more accurate population taxonomic identification. Thus, rRNA methods may be more successful than mRNA methods, especially for low-biomass samples. However, there is no absolute correlation between rRNA concentration and cell activity or growth rate, and the relationship between rRNA and cell status may vary within or between populations. Furthermore, RNA is challenging to handle. For low biomass studies, it is particularly important to take into account sample loss under extreme conditions.

Methods for targeting DNAs

Methods based on the application of ethidium monoazide bromide

Ethidium monoazide bromide (EMA) is a DNA-intercalating dye that selectively penetrates the damaged cell membrane of dead bacteria and covalently links to DNA through photoactivation. In 2003, Nogva et al. proposed a novel method for detecting living cells by applying EMA to viable PCR (v-PCR) [86]. Microbial samples are treated with a nucleic acid intercalation dye that selectively enters cells with damaged cell membranes, while intact cell membranes pose a barrier to the molecule. Once inside the dead cell, the dye intercalates into the cell's DNA. Due to the presence of an

Table 1 Viable *Salmonella* detection methods for targeting RNAs

Microorganisms	Methods	Target genes	Detection limits	Samples	References
Salmonella enterica	CRISPR/Cas13-based NASBA	16S rRNA	~1 CFU or 1% viable *Salmonella*	Cecum, colon, and rectum	[59]
Salmonella spp.	FISH	23S rRNA	1×10^5 ~ 1×10^6 CFU mL^{-1}	Artificially contaminated water and milk	[61]
Salmonella Enteritidis	Directing qPCR	16S and 23S rRNAs	2.5 log CFU per PCR	Milk	[63]
Salmonella enterica	NASBA	*dnaK* mRNA	–	–	[68]
Salmonella enterica	RT-qPCR	*sigDE* mRNA	10^0 CFU mL^{-1}	Egg broth and milk	[69]
Salmonella spp.	NASBA	*xcd* mRNA	9.5×10^3 CFU mL^{-1}	Pork background microbiota	[70]
			10 CFU 25 g^{-1} (mL)	Pork, beef, and milk	
Salmonella spp.	RT-qPCR	*invA* mRNA	40 copies per reaction	Spinach, tomatoes, peppers, and peppers	[71]
Salmonella enterica	RT-qPCR	*invA* mRNA	10^4 CFU 25 g^{-1} (~10^2 CFU g^{-1})	Jalapeño and serrano peppers	[72]
Salmonella spp.	RT-qPCR	*invA* mRNA	10^2 ~ 10^3 CFU mL^{-1}	Fresh produce	[73]
Salmonella enterica	RT-qPCR	tmRNA	1 CFU mL^{-1}	Bacteria culture	[76]
			10 CFU g^{-1}	Fresh-cut vegetables (6-h enrichment)	
Salmonella typhi	RT-PCR combined with DNA microarray	*tyv* mRNA	–	–	[85]
Salmonella spp.	RT-qPCR	*invA* mRNA	10^2 MPN[a] per reaction	Sludge	[77]
Salmonella Typhi	mPCR	*tyv* RNAs	30 cells	Contaminated water	[83]
Salmonella Enteritidis	RT-qPCR	*invA* RNA	10^7 CFU 25 mL^{-1}	Liquid whole eggs	[84]
			10^0 CFU 25 mL^{-1}	Eggs after 16-h enrichment	

[a]MPN indicates the most probable number

azide group, photolysis converts it into a highly reactive nitrite radical after exposure to strong visible light, which can react with organic molecules in its vicinity [87]. Considering the spatial proximity of the intercalating dye, it can be assumed that a reaction with DNA is likely to occur. Studies have shown that such modifications strongly inhibit DNA amplification [88, 89]. While cross-linking with DNA occurs, any unbound excess dye reacts with water molecules. The resulting hydroxylamine is no longer active, preventing the dye from reacting with DNA extracted from intact cells [55]. Soejima et al. reported that treatment with EMA followed by visible light irradiation would result in direct cleavage of chromosomal DNA from dead bacteria [90]. This hypothesis was well supported by the fact that electron microscope images showed varying degrees of DNA fragmentation upon exposure to increasing concentrations of EMA. For obvious reasons, fragmentation interferes with amplification. Typically, the EMA-PCR reduces the detection of dead bacteria by up to 3.5 logs compared to methods that do not use EMA; however, if dead Gram-negative bacteria (e.g., total coliforms) are present in large numbers in the sample, this difference may still insufficient to inhibit the amplification of their DNA. As a result, errors in results may occur.

The EMA-PCR method established by Wang et al. allowed the detection of 10 CFU mL^{-1} of viable *Salmonella* in poultry products after enrichment [91]. For rapid detection of active *Salmonella*, Lu et al. developed an EMA-LAMP method by targeting the *invA* gene [92]. The results showed that the method had high specificity and could detect as little as 100 fg of DNA.

It has been suggested that EMA may penetrate bacterial cells with intact membranes, leading to an underestimation of the number of viable cells. The extent of EMA uptake by intact cells has been reported to depend on the bacterial species and the density of the bacterial suspension. The dye may be considered insufficient to distinguish between live and dead cells for species whose intact cell membranes are not sufficiently resistant to EMA.

Methods based on the application of propidium monoazide

A promising strategy for assessing microbial viability relies on using membrane-impermeable DNA intercalation dyes as a sample pre-treatment prior to performing molecular techniques, also known as v-PCR. This method was first described by Nogva et al. in 2003 and refined by

Nocker et al. in 2006 to establish a v-PCR method using propidium monoazide (PMA) instead of EMA [86, 88, 93]. PMA has a similar mechanism of action to EMA and is considered to be a more suitable alternative to EMA with a much higher specificity for viable cells (Fig. 4). The increased specificity of PMA for living cells is thought to be mainly due to the higher charge of PMA (EMA has one positive direction compared to PMA's two). Therefore, according to the reports available, PMA has been used more than EMA in detecting viable *Salmonella* spp. (Table 2).

Using the property that PMA treatment effectively prevents PCR amplification of heat-dead *Salmonella* cells in ice cream, Wang et al. established a PMA-qPCR assay that selectively detects viable *Salmonella* down to 10^3 CFU mL^{-1}. [94] After 18 h of pre-enrichment, this assay allowed the detection of 10^0 CFU mL^{-1} of viable *Salmonella* and avoided false positive results for dead cells. Li et al. used PMA-PCR targeting *invA* to detect 30 CFU g^{-1} of viable *Salmonella* cells from enriched spiked spinach samples as early as 4 h [95].

Liang et al. applied PMA to establish a multiplex real-time quantitative PCR (mRT-qPCR) for the simultaneous detection of viable *Salmonella*, *Escherichia coli*, and *Staphylococcus aureus* [96]. The recoveries ranged from 95.7% to 105.6%. For the rapid detection of *Salmonella* Typhimurium, *Escherichia coli* O157:H7, and *Listeria monocytogenes* in vivo, Yang et al. developed a novel assay based on immunomagnetic separation (IMS) [97]. The IMS-PMA-mPCR assay was able to detect *S*. Typhimurium at a detection limit of 1.2×10^2 CFU mL^{-1}.

Salmonella is a common pathogen in raw milk. Traditional isothermal amplification methods cannot distinguish between surviving and dead bacteria, which may lead to false positive results or overestimating the number of surviving bacteria. Youn et al. reported an isothermal amplification method targeting *invA* combined with a simple sodium monoazide treatment (PMA-LAMP) for the simple and rapid detection and quantification of live *Salmonella* in rinse water [98]. The detection limit was tenfold lower than conventional PMA-PCR. Han et al. established PMA-LAMP targeting the *agfA* gene to detect live *Salmonella enterica* within 30 min and achieved sensitivity and quantification comparable to PMA-qPCR assays [99]. Based on a similar strategy, the PMA-LAMP method established by Chen et al. had a detection limit as low as 3.4 to 34 viable cells in pure culture [100]. Trieu et al. constructed an origami microdevice based on the PMA application that integrates DNA purification, LAMP, and colorimetric detection to detect active *Salmonella* immediately [101]. In addition, Chen et al. developed a real-time and visual method for detecting viable *Salmonella* in milk using competitive annealing mediated isothermal amplification (CAMP) in combination with PMA. Positive results could be directly observed by a colorimetric change from purple to sky blue. Real-time PMA-CAMP was used to quantitatively detect viable *Salmonella* in spiked milk samples with a detection limit of 10^2 CFU mL^{-1} and a recovery of 80–106%. Also based on an isothermal amplification

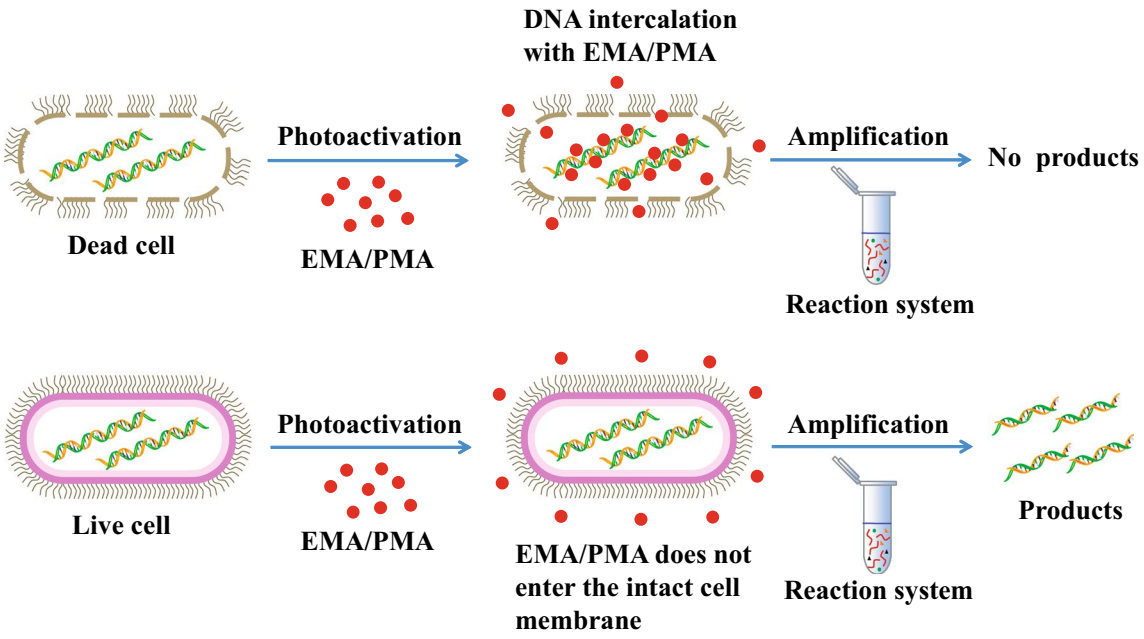


Fig. 4 Mechanism of the action of EMA/PMA [15]. The dye enters compromised or dead cells to covalently bind to DNA and prevents DNA amplification in the reaction system

Table 2 EMA/PMA-based methods for the detection of viable *Salmonella*

Dyes	Techniques used	Organisms detected	Detection limits	Samples	References
EMA	qPCR	*Salmonella*	10 CFU mL^{-1}	Chicken rinses and egg broth	[91]
	LAMP	*Salmonella*	100 fg of DNA	–	[92]
PMA	qPCR	*Salmonella*	10^0 CFU mL^{-1}	Ice cream	[94]
	Multiplex PCR	*Salmonella* Enteritidis	7.4×10^2 CFU mL^{-1}	Pure culture	[104]
			7 CFU mL^{-1}	Spiked pure milk (7-h enrichment)	
	qPCR	*Salmonella* spp.	30 CFU g^{-1}	Spinach samples	[95]
	M-qPCR	*Salmonella* spp.	10^2 CFU mL^{-1}	Bird's nest, donkey-hide gelatin, and wolfberry	[96]
	IMS-mPCR	*Salmonella* Typhimurium	1.2×10^2 CFU mL^{-1}	Pure culture	[97]
			5.1×10^3 CFU g^{-1}	Spiking lettuce, tomato, and ground beef	
	SDS-PMA-mRT-PCR	*Salmonella* Typhimurium	10 CFU mL^{-1}	Spiked milk	[103]
	LAMP	*Salmonella*	8.0×10^1 CFU per reaction	Pure culture and chicken carcasses	[98]
	LAMP	*Salmonella enterica*	4.6 CFU per reaction	Pure culture	[99]
			1.05×10^4 or 1.05×10^5 CFU g^{-1}	Spiked tomato, lettuce, and spinach	
	LAMP	*Salmonella*	3.4–34 cells	Pure culture	[100]
			6.1×10^4 CFU g^{-1}	Spiked produce samples	
	LAMP	*Salmonella* spp.	14 CFU mL^{-1}	Chicken meat supernatants	[105]
	CAMP	*Salmonella*	10^2 CFU mL^{-1}	Spiked milk samples	[106]
	CPA	VBNC *Salmonella*	10^3 CFU mL^{-1}	Rice food products	[102]

strategy, Ou et al. developed a propidium monoazide-crossing priming amplification (PMA-CPA) method targeting the *invA* gene for the specific detection of VBNC *Salmonella* in food samples [102].

For rapid and sensitive detection of surviving *Salmonella* Typhimurium in milk, Shi et al. used IMS to isolate target bacteria and combined treatment with SDS and PMA before amplification, which effectively eliminated false positive results from dead bacteria and identified surviving target bacteria with good sensitivity and specificity [103].

Despite the advantages of viable PCR, there is evidence that v-PCR using DNA interpolation dyes has practical and theoretical limitations, especially when applied to environmental samples [107–109]. A comparative study by Nocker et al. provided early evidence of differences in the specificity of the two dyes [93]. It was shown that EMA could enter and successfully stain living bacterial cells such as *E. coli* O157:H7, *Listeria monocytogenes*, and *Staphylococcus aureus*, while PMA showed better specificity. Lee et al. found that EMA penetrated heat-exposed cells more effectively than PMA, resulting in a more effective inhibition of DNA amplification in heat-damaged cells by EMA [54]. Additionally, PMA was found to be ineffective in differentiating low numbers of live cells from dead heat cells in the gut [110].

The primary strategy for reducing the uptake of EMA by living cells is to reduce the concentration of the dye, resulting in fewer dye molecules entering the living cells. This approach is made possible by the ability of EMA to effectively penetrate dead cells even at relatively low dye concentrations. Most current studies mainly used 10 μg mL^{-1} as the preferred concentration of EMA, while higher concentrations lead to penetration of live cells [111–113]. In addition, it has also been suggested that the minimum amount of EMA required to inhibit dead cell signaling effectively is 2.5 μg mL^{-1} [114], 2.3 μg mL^{-1} [115], 1.5 μg mL^{-1} [116], and 1 μg mL^{-1} [54]. In addition to minimizing EMA concentrations, an increasing number of studies have reported incubating dye on ice or at a low temperature (4 °C) rather than at room temperature.

To improve the limitations of PMA in excluding dead cell signals, the main strategy is to amplify longer DNA sequences. It has been shown that quantitative PCR (qPCR) amplification of short DNA fragments (< 200 bp) after PMA treatment resulted in incomplete suppression of the signal from heat-treated *Salmonella enterica*. In contrast, PCR amplification of long DNA fragments (1.5–1.6 kb) completely suppressed the dead cell signal [117]. Other potential strategies may include treating the sample with a moderate membrane stabilizer, exposing the sample at higher a temperature, and considering applying higher dye concentrations. PMA improved structure PMAxx as an alternative to PMA is also a good option. One study showed that PMAxx-based qPCR is more sensitive in quantifying viability compared to culture methods and is suitable for quantifying the viable and non-viable load of *Salmonella*

from poultry environment [118]. In addition, the use of enrichment methods before PMA-PCR analysis was also beneficial to improve the accuracy of viable bacteria detection [94, 119].

Other optional DNA-based assays

Yin et al. have developed a method for rapidly detecting total live bacterial cells in a wide range of species using a newly developed handheld fluorometer and the fluorescent dye Calcein UltraGreen™ AM [120]. The portable rapid detection fluorometer has a wide dynamic range of relative fluorescence intensities, detecting 10^5 to 10^{10} cells mL^{-1}, and does not require pre-culture. The results of the method were compared with those of the plate count method with a relative standard deviation of less than 6%, demonstrating its suitability for accurate and rapid detection of viable bacterial cell counts in a wide range of samples.

In addition, capturing viable *Salmonella* cells using anti-*Salmonella* antibody-modified magnetic beads allows the removal of the yolk component that inhibits PCR. The collected live *Salmonella* cells could be rapidly detected by PCR targeting the *invA* gene on a microfluidic chip device. The method could detect at least 5×10^4 cells mL^{-1} within 6 h [121].

PagC is a component of membrane vesicles and acts by inhibiting the proliferation of *Salmonella* in macrophages [122]. Xu et al. found that the *pagC* gene is overexpressed in H_2O_2-induced *Salmonella* in the VBNC state and is expected to be used as a new biomarker for the detection of VBNC *Salmonella* [123].

Zhai et al. developed a molecular beacon method based on a duplex real-time nucleic acid sequence-based amplification (real-time NASBA) assay to simultaneously detect viable *Salmonella* spp. and *S*. Paratyphi C cells in retail food by targeting the SPC_0908 and *xcd* genes [124].

Zhang et al. achieved a sensitive detection of viable *S*. Enteritidis down to 60 CFU mL^{-1} with a linear range of 10^2 to 10^7 CFU mL^{-1} by coupling a cascaded two-stage target sequence-recyclable toehold strand displacement with aptamer-based target recognition [125].

For detecting viable *S*. Typhimurium directly, Zhang et al. designed an intelligent sensor system using scaffold silver nanoclusters (AgNCs) generated from a triple trigger sequence-regenerated strand displacement amplification and a self-protecting hairpin template [126]. In the presence of viable *S*. Typhimurium, replication of the trigger sequence and sequential production of the scaffold in cascade was achieved, forming highly fluorescent AgNCs that provided a significantly enhanced fluorescence signal, enabling ultra-sensitive detection of live *S*. Typhimurium down to 50 CFU mL^{-1} with a linear range of 10^2–10^7 CFU mL^{-1}. To further simplify the steps and increase the efficiency of the assay, ultra-sensitive detection of live *Salmonella* down to 25 CFU mL^{-1} with a linear range of 50 to 10^4 CFU mL^{-1} was achieved based on the previous study and self-protection mediated by CuNPs hairpin scaffolds [127].

In addition, Labib et al. developed a highly selective aptamer-based bacterial viability impedance sensor that can successfully detect viable *S*. Typhimurium down to 600 CFU mL^{-1} [128]. Chen et al. used dual aptamers to capture *S*. Typhimurium and PCR products individually [129]. The stable structure formed between the amplicons and AuNPs-probe prevented self-aggregation and discoloration of AuNPs in solution. The method enabled visual detection and had a low detection limit of 33 CFU mL^{-1} for *S*. Typhimurium.

Quintela et al. achieved simultaneous colorimetric detection of 19 *Salmonella* species using an optical biosensing platform with oligonucleotide-functionalized AuNPs [130]. The method was 100% specific and had a detection limit of less than 10 CFU mL^{-1} for the target bacteria in both pure cultures and complex matrices.

Phage-based strategies

The high specificity and natural affinity of phages for their host bacteria make phage-based methods attractive. Pages can only replicate within living bacterial cells, so phage-based methods can be optional for detecting live bacteria [131]. Phage-based assays primarily employ phagocytic phages as lytic agents to detect new progeny phages or intracellular material released from target bacterial cells to indicate cell viability. One of the simplest phage-based tests is known as the phage amplification assay [132]. The technique is based on the phage-phagocytosis cycle, with bacterial spot formation as the endpoint of detection. Due to the simplicity and rapidity of the method, it is currently used to detect foodborne pathogens such as *Salmonella* Typhimurium, *Staphylococcus aureus*, and *Salmonella* Enteritidis [132–135]. Also, phage-based detection can be improved by combining the cellular part of the plaque assay with other methods, such as immunological or molecular techniques to detect progeny phage or phage DNA (Fig. 5) [136–139].

A phage amplification-based analysis (PAA) method based on the isolated *Salmonella* phage T156 was successfully applied to quantify viable *Salmonella* in food matrices such as milk by Huang et al. The method had a detection limit of 1 CFU mL^{-1} and could detect only live bacteria [140]. When combined with qPCR, PAA-qPCR further reduces the detection time from 6.5 h to 3.5 h with a detection limit of 10 CFU mL^{-1}. In addition, the initial bacterial concentration can also be deduced by monitoring the number of compounds released after phage lysis by bioluminescence using enzymes and substrates. In recent years, genetically

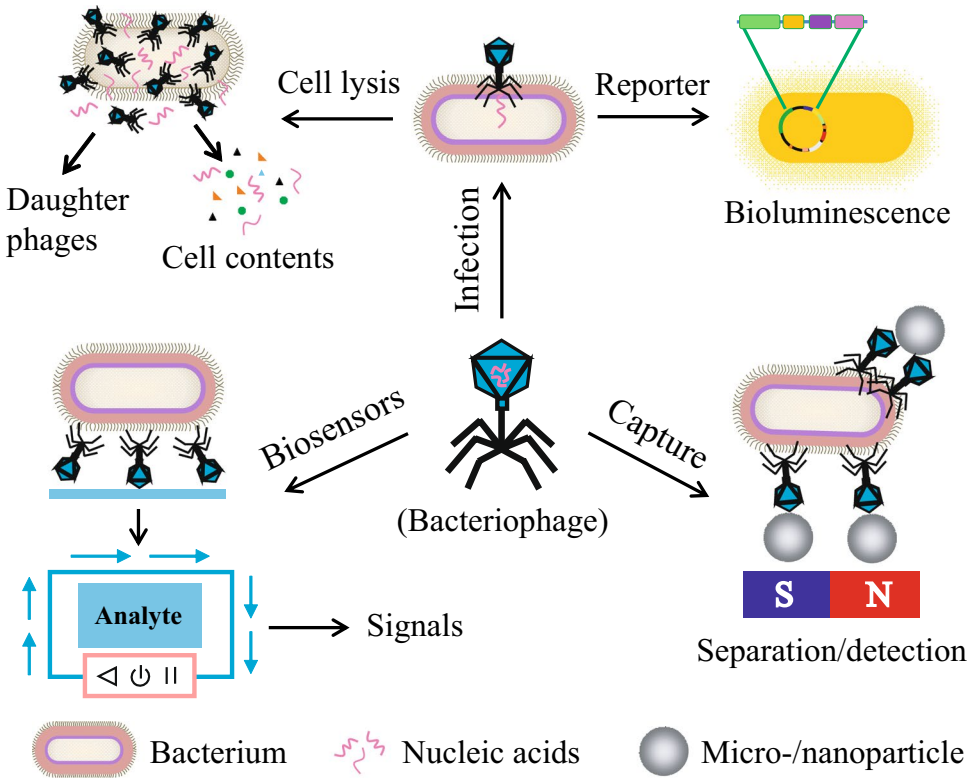


Fig. 5 Phage-based bacterial detection methods [131]. When phages infect a target bacterium and cause it to lyse, daughter phages or cell contents can be detected. Alternatively, phages can be used to mark hosts with fluorescent, colorimetric, or optical markers after the introduction of exogenous genes into bacteria. In addition, phages can also be used to construct biosensors, and phages attached to micro-nanoparticles can be used as capture elements in different assays

engineered lysogenic phages containing luciferin, proteases, and alkaline phosphatases have been reported [141–143]. As the released cell contents are highly conductive, changes in conductivity in the environment can be used as a signal for the presence of live bacteria in the sample. Conductivity changes caused by lysed bacteria can be detected using impedance spectroscopy in combination with a microchip. Some of these phage-based assays have proven helpful in detecting live bacteria in broth cultures, drinking water, etc. [144, 145].

The bioluminescent reporter phage is designed and constructed by transducing a luciferase gene in the genome. Relying on the host specificity of the phage, the system enables rapid, sensitive, and specific detection of live bacteria [146]. Chen and Griffiths used three phages to detect *Salmonella* at 10 CFU mL^{-1} from the pre-enriched broth for 6 h and directly from contaminated whole eggs within 24 h [147]. Thouand et al. reported a P22::luxAB assay for the detection of *Salmonella* in poultry samples with a detection limit up to $10^2 \sim 10^4$ CFU mL^{-1} [148]. Using phages to express NanoLuc, Nguyen et al. developed a method for the detection of *Salmonella* using luciferase reporter phages [149]. The method had a detection limit of $10 \sim 100$ CFU mL^{-1} without enrichment. Kim et al. developed a bioluminescent reporter phage SPC32H-CDABE by inserting the bacterial luxCDABE operon into the *Salmonella* temperate phage SPC32H genome [150]. LuxCDABE operon provided both luciferase and its substrate, eliminating the need for a substrate addition step. At least 20 CFU mL^{-1} of *S.* Typhimurium could be detected within 2 h using SPC32H-CDABE.

The detection limits of bioluminescent reporter phages can be increased significantly if immunomagnetic separation techniques are used in the pre-enrichment of target bacteria. It is particularly suitable for the rapid and efficient enrichment of pathogens using the cell wall-binding domain of phage endolysin coated on paramagnetic beads [151, 152]. Pre-enrichment of target bacteria by magnetic separation followed by detection with bioluminescent reporter phages reduced the detection limit by several orders of magnitude and allowed for the hypersensitive diagnosis of live bacteria only [153]. Using an immunomagnetic separation method, Favrin et al. was able to detect *Salmonella enterica* as low as 3 CFU 25 g^{-1} of food [154].

Zhao et al. developed a phage-based bio-orthogonal reaction-amplified microparticle counting sensing method to detect viable *Salmonella* in different food products rapidly [155]. By introducing a bio-orthogonal reaction to further amplify the signal, the presence of *Salmonella* could specifically induce quantitative changes in functionalized polystyrene microspheres, which could be monitored by a microporous resistance particle counter. This sensor had a detection limit of 33.58 CFU mL^{-1} and a linear range of 10^2 to 10^6 CFU mL^{-1}.

Despite the apparent advantages of phage-based assays, such methods have yet to be widely used. A major reason is the need for sufficiently specific but broad host-range phages.

If not, they need to be isolated from the environment, which would be time-consuming and uncertain. In particular, it is difficult to find a specific phage in closely related organisms such as *Salmonella*, *Shigella*, and *E. coli*. Appropriate passaging manipulation of the selected phage without affecting its life cycle and ability to infect the host may be another challenge. As new DNA sequencing technologies make the genome sequences of phages and bacterial hosts increasingly available, phage-based approaches will be further developed, especially when targeting difficult-to-detect bacterial species. In addition, combining efficient enrichment and immobilization techniques with bioluminescent phage detection provides microbiologists with powerful diagnostic tools for specifically detecting bacterial pathogens.

Biosensor-based strategies

A biosensor is a device that converts a signal from biology into an electrochemical, optical, or another physical signal. It has the advantages of high detection sensitivity, fast response time, automation, and miniaturization (Fig. 6) [156]. These promising features make biosensors ideally suited for immediately detecting pathogens in the field. In addition, by combining biosensors with molecular biology techniques, target sequences in the genome of pathogenic microorganisms can be precisely identified to enhance target detection performance and range effectively.

In the context of viable bacteria detection, biosensors can bind microorganisms to specific biological recognition molecules (e.g., antibodies, DNA probes, etc.) to produce a recognition signal that can be converted into an electrical signal for detection [157]. A fluorine-doped tin oxide (FTO) electrode based on reduced graphene oxide (rGO) was fabricated by Mahari et al. [158]. Then rGO was labeled by *S.* Gallinarum and *S.* Pullorum-Ab via carbodiimide activation. The immunosensor exhibited a linear detection range ($1–1 \times 10^5$ cells) with detection limits of 37 and 25 for viable *S.* Gallinarum and *S.* Pullorum, respectively, and could be used for rapid detection of salmonellosis in meat and fecal samples. The laser-induced graphene (LIG) biosensor constructed by Soares et al. using an antibody-functionalized LIG electrode enabled the detection of viable *Salmonella* in 22 min without pre-culture [159]. The results showed that the sensor could detect live *Salmonella* in chicken broth over a wide linear range ($25–10^5$ CFU mL^{-1}) with a low detection limit (13 ± 7 CFU mL^{-1}) and showed high selectivity. Liu et al. proposed an impedance-based microfluidic biosensor with two sensing zones consisting of an interdigitated electrode array of 50 finger pairs each [160]. The sensor could detect *Salmonella* serotypes B and D within 1 h with a detection limit as low as 300 cells mL^{-1}. In addition, the sensor could differentiate between low concentrations of live *Salmonella* cells and high concentrations of dead *Salmonella* cells.

Biosensors represent an attractive method for the rapid detection of bacteria. Angelopoulou et al. presented an optical biosensor based on white light reflectance spectroscopy for the detection of *Salmonella* Typhimurium in drinking water [161]. A mixture of pre-incubated bacteriolytic and anti-*Salmonella* lipopolysaccharide antibodies was pumped onto a chip, and the signal was then enhanced with biotinylated secondary antibodies and streptavidin. A minimum of 320 CFU mL^{-1} of viable *Salmonella* could be detected within 15 min using this sensor.

Target-induced aptamer displacement on AuNPs deposited electrode with rolling circle amplification (RCA) facilitated the reproducibility of sensor detection and reduced matrix effects. Ge et al. hybridized RCA products from

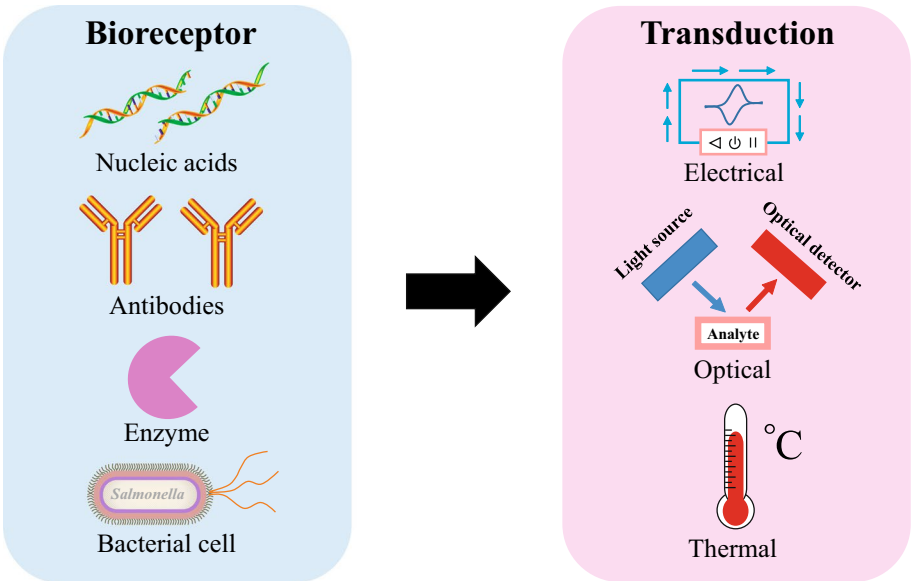


Fig. 6 Schematic diagram of the principle of biosensors based on different transduction methods [156]

Salmonella Typhimurium with biotinylated probes and used an enzymatically amplified electrochemical sensor for interpretation [162]. The electrochemical sensor had a detection limit as low as 16 CFU mL^{-1} and a linear detection range of 20 to 2×10^8 CFU mL^{-1}.

Other testing methods available

To eliminate the enrichment step of overnight incubation and detect trace amounts of pathogens within one day, Murakami et al. developed a filter-based pathogen enrichment method using a unique combination of glass fiber depth filters and porous filter aid materials, which could specifically detect 1 CFU mL^{-1} of viable *Salmonella enterica* in food [163].

Desmonts et al. demonstrated that the indirect fluorescent antibody (IFA) technique could detect VBNC *Salmonella* in environmental water systems and had advantages over culture methods [164]. The detection limit of the IFA method was as low as 7.5×10^3 cells mL^{-1} of wastewater.

Using the protective effect of egg white proteins and peptones, proteolytic enzymes do not attack living cells when hydrolyzing the egg white proteins responsible for fouling. Based on this, Ku et al. report a method for concentrating bacteria by microfiltration that detects less than 13 CFU of *Salmonella* per 25 g of egg white [165]. A combination of enzyme treatment, controlled cross-flow on both sides of the hollow fibers, and medium selection was vital in preventing membrane fouling, resulting in rapid concentration and subsequent detection of low numbers of microbial cells.

D'Urso et al. used an identification buffer (including 4 M guanidine thiocyanate, 2 M NaCl, and 25 mM Tris–HCl) mixed with the sample to be tested and then incubated to ensure that all dead cells passed through the filter [166]. By resuspending the filter, viable *Salmonella* can be detected and combined with qPCR for rapid detection with reasonable accuracy. Similarly, Vibbert et al. used 0.2 µm cutoff polysulfone and polyethersulfone hollow fiber membranes to recover and detect 1–10 CFU mL^{-1} of viable *Salmonella* from 400 mL of chicken rinse [167].

Based on an optimized Nanopore sample extraction and library preparation protocol, Yang et al. developed a novel strategy for real-time multiplex identification of viable pathogens in food by direct metatranscriptomic RNA-seq and multiplex RT-PCR amplicon sequencing on the Nanopore MinION [168]. This assay was validated by *Salmonella* Enteritidis et al. and demonstrated high sensitivity and accuracy.

To dispense with the use of chemically labeled RNA substrates, Zhang et al. used a luminescent RNA inducer, Broccoli, as a subunit of activated CRISPR-Cas13a to detect pathogenic RNA [169]. CRISPR-Cas13a allowed precise differentiation between live and dead bacteria, with a detection limit down to 10 CFU of live pathogenic bacteria.

Phenotypes of bacteria, such as drug resistance and viability, are difficult to obtain quickly. An allele-specific isothermal RNA amplification (AlleRNA) method, based on NASBA and combined with an amplification refractory mutation system, was established by Liao et al. [170]. This method had a detection limit of 80 CFU for live quinolone-resistant *Salmonella enterica*.

Fourier transform infrared (FT-IR) spectroscopy allows rapid detection, quantification, and classification of microorganisms, spoilage, and pathogenic bacteria with little or no sample pre-treatment. This technique can be used for strain-level identification and to distinguish between live, sub-lethally damaged and dead bacterial cells [171]. Davis et al. combined FT-IR analysis with IMS to rapidly detect and differentiate *Salmonella enterica* isolated from chicken meat [172]. The detection limit of the developed IMS-FT-IR method was 10^4 CFU g^{-1}. The method also detected live cells in the presence of dead cells with results comparable to those obtained with the LIVE/DEAD BacLight™ Bacterial Viability Kit.

Laser-induced breakdown spectroscopy (LIBS) is an ideal candidate for rapidly detecting biological contaminants on food and food processing surfaces due to the low sample size and immediate results. Multiple regression analysis based on LIBS data by Multari et al. could accurately distinguish between *E. coli* O157:H7 and *Salmonella enterica* and their metabolic status (viable or heat-killed) [173].

Conclusions

Much of applied microbiology research and quality control is related to whether cells are alive or dead. There is a constant search for faster and more sensitive methods to detect viable bacteria. Culture-based methods are laborious and time-consuming, and relatively limited in their ability to detect bacteria in the VBNC state if they are present in the sample. Molecular assays, particularly RNA-based tests, are a potential solution for rapidly detecting viable bacteria. However, the perishable nature of RNA remains an obstacle to the large-scale application of viable *Salmonella* detection. The principle of live-dead distinction in v-PCR is based on the integrity of the membrane. Although this conservative indicator of viability is commonly used, it has limitations. The diagnostic method is not applicable to cells subjected to sterilization or disinfection treatments. In contrast, loss of redox and esterase activity tends to correlate better with loss of culturability. Phage-based methods show high sensitivity for detecting viable bacteria in different matrices. Various emerging methods developed

in recent years provide new tools to support the rapid and accurate detection of viable *Salmonella* and have significant scope for development.

The VBNC state is a survival strategy adopted by bacteria to cope with adverse environmental conditions. Bacteria in the VBNC state lose their ability to culture on microbial media, but retain their cellular integrity and viability in terms of respiration, enzyme activity and gene expression. With the continuous research on bacteria in VBNC status, there are more and more methods used for VBNC bacteria detection, such as fluorescence microscopy detection techniques (including live bacteria direct counting method, LIVE/DEAD BacLight™ Bacterial Viability Kit), flow cytometry, nucleic acid-based detection methods, and immunological detection methods. However, in the actual testing work, there is still a lack of criteria and corresponding testing methods to fundamentally determine whether the bacteria have entered the VBNC state. In recent years, the use of molecular biology methods and the combination of technologies to detect bacteria in VBNC status has become a new trend, which can effectively avoid the shortcomings of single detection methods and improve the accuracy of detection. This will provide a more effective method and approach for studying VBNC *Salmonella*.

In the future, the methods described above will mature and provide us with more possibilities to detect viable *Salmonella* populations. Certainly, more rapid and sensitive methods for detecting viable *Salmonella* are also imperative. In conclusion, research on strategies for detecting viable *Salmonella* is of great importance and relevance to the scientific prevention and control of potential risks to humans and animals.

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Data Availability Statement Not applicable.

Declarations

Conflict of interest There are no conflicts of interest to declare.

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