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Specific detection of viable *Salmonella* Enteritidis by phage amplification combined with qPCR (PAA-qPCR) in spiked chicken meat samples

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ABSTRACT

Serovar Enteritidis represents 45.7% of all *Salmonella* reported human cases identified in Europe. Additionally, "minced meat and meat preparations from poultry" have a high level of non-compliance, regarding *Salmonella* regulation.

In the current study, a novel method based on the amplification of the *Salmonella* bacteriophage vB_SenS_PVP-SE2, coupled with real-time PCR (qPCR), was developed and evaluated, for the rapid detection of viable *Salmonella* Enteritidis in chicken samples. The results obtained indicated that the qPCR method could detect down to 0.22 fg/μL of pure virus DNA and a concentration of viral particles of 10^3 pfu/mL. After a short bacterial recovery step, the addition of bacteriophages to spiked chicken samples indicated that 8 cfu/25 g could be detected within 10 h, including the time for DNA extraction and qPCR analysis. Additionally, the evaluation of the performance parameters: relative sensitivity, specificity, accuracy, positive and negative predictive values, and index kappa of concordance, obtained values higher than 92%, and the acceptability limit values were within the limits. All these results demonstrate that the proposed methodology is a powerful tool for the rapid detection of viable *Salmonella* Enteritidis.

1. Introduction

The genus *Salmonella* comprises two species (*Salmonella enterica* and *Salmonella bongori*) and more than 2500 different serovars (Grimont & Weill, 2007). This genus is one of the most common foodborne pathogens worldwide, as highlighted by the fact that in 2015, 94 625 cases of salmonellosis were reported in Europe, representing about 28% of all reported foodborne diseases in Europe, and a 1.9% increase with respect to 2014; furthermore, ten member states reported 126 fatal cases (EFSA and ECDC, 2017).

It has been extensively reported that the traditional methods for the detection of foodborne pathogens are lengthy and laborious. For instance, those described by the International Organization for Standardization (ISO) and the Bacteriological Analytical Manual (BAM) from the U.S. Food and Drug Administration (Andrews, Jacobson, & Hammack, 2011; ISO, 2003) require several hands-on steps over several days (from three to six with confirmation). Against the classical approaches, molecular methods have arisen as fast and reliable alternatives. Focus has been put mainly on those based on nucleic acids amplification, such as the Polymerase Chain Reaction or the real-time PCR (PCR/qPCR, (Chapela, Garrido-Maestu, & Cabado, 2015)), and more recently those based on isothermal amplification such as Loop-mediated isothermal amplification (LAMP, (D'Agostino, Diez-Valcarce, Robles, Losilla-Garcia, & Cook, 2015)), Ligase Chain Reaction (LCR (Jang et al., 2003),), or Recombinase Polymerase Amplification (RPA, (Kim & Lee, 2016)). However, a drawback commonly attributed to these techniques is their incapacity to differentiate between viable and non-viable microorganisms. Efforts to overcome this limitation have resulted in the development of alternative approaches that lead to the specific detection of viable bacteria, such as amplification coupled with propidium monoazide (PMA) treatment, RNA amplification, or specific sample treatments to eliminate non-viable microorganisms, thus allowing the direct application of conventional nucleic acid amplification techniques (D'Urso et al., 2009; Feng et al., 2016; Zhang, Brown, &

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González-Escalona, 2011).

An entirely different approach for the assessment of bacterial viability was reported by Stewart at al., (Stewart et al., 1998), who developed the Phage Amplification Assay (PAA), in which the bacterial viability is inferred from the observation of phage plaques within a bacterial lawn, after incubating a sample containing the target bacteria with a high titer of a suitable bacteriophage.

(Bacterio)phages are viruses that specifically infect bacteria and thus have been considered exceptional tools for pathogen detection. Also, their lytic cycle usually takes only 1–2 h, and their multiplication within the cell works as an "enrichment" step allowing to shorten detection times (Schmelcher & Loessner, 2014).

The combination of PAA with qPCR has been reported previously for the detection of *Bacillus anthracis* and *Ralstonia solanacearum* (Kutin, Alvarez, & Jenkins, 2009; Reiman, Atchley, & Voorhees, 2007). However, it has not been explored in depth for the detection of foodborne pathogens; thus, the aim of the present study was to develop and evaluate a fast method based on PAA-qPCR for the detection of viable *Salmonella enterica* serovar Enteritidis (*S*E) in chicken samples.

2. Materials and methods

2.1. Bacterial strains, bacteriophage and culture media

Salmonella Enteritidis S1400 a wild strain isolated from poultry, that belongs to the private collection of the University of Bristol (Sillankorva et al., 2010), was selected as the reference strain For sample pre-enrichment, as well as for bacterial dilutions, Buffered Peptone Water (BPW, Biokar diagnostics S.A., France) was used. Regarding solid media, *S*E was plated on Luria-Bertani agar (LB, Sigma-Aldrich, St. Louis, USA). All enrichments, and plate incubation steps were performed at 37 °C overnight unless otherwise specified.

Confirmation of the presence of *S*E in spiked food samples after pre-enrichment, was performed by streaking the pre-enriched samples on Xylose Lysine Desoxycholate Agar (XLD, Biokar diagnostics S.A., France). The plates were incubated at 37 °C overnight.

The *Salmonella* phage vB_SenS_PVP-SE2 (GenBank accession no. MF431252.1), previously named ϕ38, isolated by Sillankorva et al., (Sillankorva et al., 2010), was chosen for the method. To determine phage concentrations (pfu/mL), ten-fold serial dilutions prepared in SM buffer (100 mM NaCl, 50 mM Tris, 8000 mM $MgSO_4{\cdot}7H_2O$, pH 7.5) were performed. Then 5 mL of molten semi-solid LB (7.5 g/L agar) containing 100 μL of an overnight culture of *S*E and 100 μL of the corresponding phage dilution were poured on solid LB. These plates were incubated at 37 °C overnight.

2.2. Primer and probe design

Sequences from the receptor-binding protein (RBP) region of *Salmonella* spp. phages were obtained from the GenBank, downloaded and aligned with CLC Sequence Viewer (C L C Bio-Qiagen, 2016). The consensus sequence was chosen for primer/probe design with Primer 3 (Untergasser et al., 2012). All primers and probes were purchased from Integrated DNA Technologies (IDT, Integrated DNA Technologies Inc., Leuven, Belgium) and Sigma-Aldrich (Sigma–Aldrich, St. Louis, USA). RBP-F: CCGAACAACAGTCTCACCGA, RBP-R: CTACAATTTTACCGGCG GCG, RBP-P: $^{56-FAM}$ AACAACAAG/^{ZEN}/GCGCGCCCGTACGA^{3IABkFQ} (IABkFQ and ZEN are a fluorophore and a quencher, respectively, are Trademarks of IDT).

2.3. DNA extraction

A simple thermal lysis DNA extraction protocol was selected for ease of use and rapidity. Briefly, 1 mL of the pre-enriched sample was taken, heated with agitation (1400 rpm) at 99 °C for 10 min in a Thermomixer comfort (Eppendorf AG, Germany). Once finished, the samples were centrifuged at 4000 g for 10 min and 4 °C. The supernatant was transferred to a new tube and stored at −20 °C until use.

2.4. qPCR

All qPCR experiments were performed in 20 μL with the following components: 10 μL of Maxima Probe/ROX qPCR Master Mix (Thermo Fisher Scientific Inc., Waltham, MA, USA), 100 nM primers and 150 nM probe for phage assays, and 2 μL of template. The thermal profile selected consisted of 2 min at 50 °C for Uracil-DNA Glycosylase (UDG) treatment (to avoid carryover contamination), followed by 10 min at 95 °C hot-start polymerase activation, and 40 cycles of dissociation at 95 °C for 15 s and annealing-extension at 63 °C for 60 s.

2.5. Evaluation of the efficiency and limit of detection of the PAA-qPCR

The assessment of the efficiency of the PAA-qPCR was determined by performing ten-fold serial dilutions of pure vB_SenS_PVP-SE2DNA in Tris-EDTA 1X (TE, 10 mM Tris-HCl, 1 mM EDTA, Sigma–Aldrich, St. Louis, USA). All dilutions were analyzed in duplicate, as described in M &M 2.4.

The limit of detection (LoD) of the PAA-qPCR for pure viruses was evaluated by performing ten-fold dilutions of vB_SenS_PVP-SE2 phages in BPW, and 2 μL of each dilution were directly analyzed, as described in M&M 2.4. The phage stock (dilution 0) was diluted 1:2 and this was used as the highest concentration.

2.6. Spiked sample preparation

Forty-one raw chicken breast samples, purchased from local supermarkets, were processed as follows: 25 g were weighed and 225 mL of 37 °C pre-warmed BPW were added, the matrix was homogenized for 30 s in a Stomacher 400 Circulator (Seward Limited, West Sussex, UK); then 1 mL of the appropriate dilution of *S*E (Table 1), prepared as mentioned above, was added and homogenized again for 30 s. This matrix was incubated for 3 h at 37 °C with agitation (120 rpm). After this initial incubation step, 10^3 - 10^4 pfu/mL vB_SenS_PVP-SE2 phages were added (concentration selected after evaluation of pure phage LoD). After carefully mixing, 1 mL was taken, being this the time 0 (T0) sample. The matrix was re-incubated, and additional samples were withdrawn after 3 h and 6 h.

Every aliquot was processed as described above in M&M 2.3 and in M&M 2.4. The PAA-qPCR cycle of quantification (Cq) values obtained after each incubation time (T3 and T6) were compared against T0 so that a Cq reduction would be related to an increase in phages' DNA, associated with their replication in viable bacteria after infection.

Table 1
Samples inoculated with *S.* Enteritidis.

Number of samples	Inoculum level (cfu/25 g)	Result
10*	8	+
3	5/10/9**	+/+/-**
5	5.2 × 10/9.8 × 10/9.4 × 10/[a]8.6 × 10	+/+/+/+[a]
5	5.2 × 10^2/9.8 × 10^2/9.4 × 10^2/[a]8.6 × 10^2	+/+/+/+[a]
2	9.8 × 103	+
2	9.8 × 10^4	+
2	9.8 × 10^5	+
6	0	–
2	[b] 8.0 × 10^7	–
2	[b] 8.0 × 10^5	–
2	[b] 8.0 × 10^3	–

*10 samples spiked for LoD evaluation.
**ND by PAA-qPCR.
[a]Two samples were spiked with the specified concentration.
[b]Concentration of non-viable bacteria.

2.7. Confirmation of viability

In order to verify that the proposed methodology only detected viable *S*E, 6 additional samples were spiked with dead *S*E. Non-viable SE were obtained as follows: fresh pure cultures were ten-fold serially diluted and plated to determine viable counts, afterwards were autoclaved for 30 min at 121 ºC to completely inactivate the bacteria. Once the treatment was completed, 10^3 10^5 and 10^7 cfu/mL of dead bacteria were added to the corresponding food sample, and processed as described above.

2.8. Evaluation of the PAA-qPCR method

The method was evaluated considering the LoD of *S*E and the following performance parameters: relative sensitivity, specificity and accuracy (SE, SP and AC), positive and negative predictive values (PPV and NPV), the kappa index of concordance (*k*) and the acceptability limit (AL). These parameters were determined by comparing the results obtained with the expected values (positive for samples spiked with viable *S*E, and negative for non-spiked, or spiked with dead bacteria).

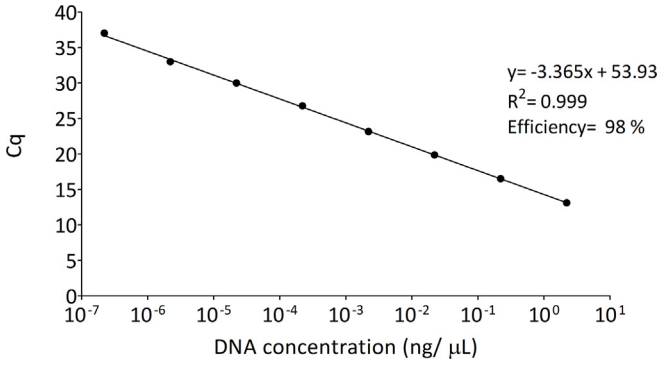
The evaluation of the LoD consisted on the determination of the lowest detectable concentration of *S*E. To this end, 10 chicken samples were spiked with low bacterial concentrations, typically ≤10 cfu/25 g and the procedure described in M&M 2.5 was followed. In order to be accepted, 90% of positive samples would have to be detected, i.e. 9 out of 10 positive samples.

Regarding the other parameters, after analysis, all samples were classified as Positive or Negative Agreement if the obtained results matched those expected (PA and NA), and Positive or Negative Deviations if the results did not match (PD and ND). With these values, the performance parameters were calculated as described in previous studies (Anderson et al., 2011; D'Agostino et al., 2016; Tomas, Rodrigo, Hernandez, & Ferrus, 2009).

3. Results

3.1. Evaluation of the efficiency and limit of detection of the PAA-qPCR

Pure phage DNA was ten-fold serially diluted, and consistent detection was achieved from 2.2 ng/μL to 0.22 fg/μL, covering eight orders of magnitude, with an amplification efficiency of 98% and a correlation coefficient of 0.999. When the same experiment was performed with pure phages, which were directly loaded into the corresponding qPCR wells, the detection was possible from 6.6×10^{11} pfu/mL down to 1.3×10^3 pfu/mL, being obtained an amplification efficiency of 99% with a correlation coefficient of 0.995. These results are graphically presented in Fig. 1a and b. Based on these results, and keeping in mind that the LoD of pure phages was 10^3 pfu/mL, it was decided to add a final concentration of 10^3 - 10^4 pfu/mL to each sample.



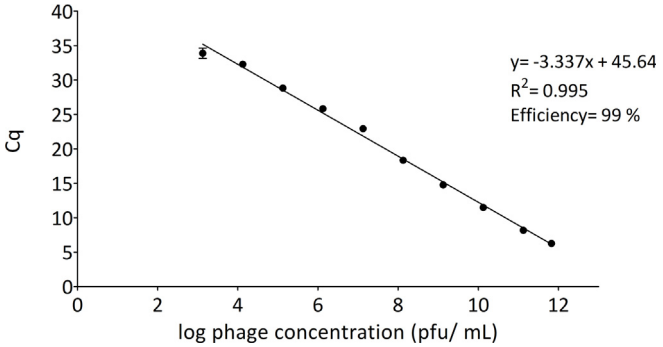


Fig. 1. (*continued*)

Fig. 1. a) Efficiency of the amplification obtained after ten-fold serially diluting pure phage vB_SenS_PVP-SE2 DNA. b) Amplification efficiency obtained after directly loading phages as the template, without prior DNA extraction.

3.2. Confirmation of viability

The inoculation of the food samples with non-viable microorganisms, even at the highest concentration (10^7 cfu/mL) did not obtain any positive result (interpreted as Cq variation after incubation with the phages). This simple experiment demonstrates that the proposed methodology only detected viable *S*E.

3.3. Evaluation of the PAA-qPCR method

The LoD of the method was determined to be < 10 cfu/25 g for a total time of analysis of 10 h, which included 3 h of pre-enrichment, 6 h of co-incubation and 1 h of DNA enrichment and qPCR analysis.

The viable plate counts indicated that the actual value was 8 cfu/25 g, but concentrations down to 5 cfu/25 g could also be detected (Table 1). It was also observed that, if the bacterial concentration was high (10^2-10^3 cfu/25 g), the detection of *S*E could be performed after 3 h of co-incubation, as shown in Fig. 2. Thus, the total analysis time could be reduced to 7 h.

The evaluation of the other performance parameters, based on the results obtained from 41 spiked samples, and after 6 h of co-incubation, revealed values of 96.6%, 100% and 97.6 for the SE, SP and AC respectively. Regarding the PPV and NPV, the values obtained were 100% and 92.3% respectively. The AL values obtained were 1-1, and most importantly, the *κ*, which measures the degree of agreement with the expected results, was 0.94 (1.0 maximum). Only one ND was observed, being this from a sample spiked with 9 cfu/25 g. No positive results were obtained from any of the negative samples, including those inoculated with different concentrations (10^3-10^7 cfu/25 g) of non-viable microorganisms, demonstrating the specificity of the assay, and that, as

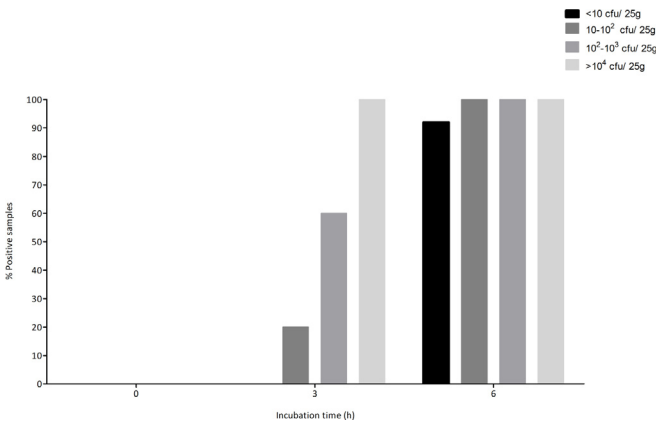


Fig. 2. Percentage of positive samples at different co-incubation times, after bacteriophage addition.

expected, only viable *S*E would be detected. These results are summarized in Table 1.

4. Discussion

In 2015, serovar Enteritidis remained as one of the most reported serovars among all *Salmonella* identified, representing 45.7% of all the identified human cases. Additionally, the highest occurrence of samples non-compliant with *Salmonella* criteria was found in foods of meat origin that were intended to be cooked before consumption, having "minced meat and meat preparations from poultry" which represents a notable level of non-compliance, among the foodstuffs analyzed (EFSA and ECDC, 2017). These data indicate that new methods, which could allow faster detection of these pathogens, are needed. Phages have been previously applied for the detection of pathogenic microorganisms, particularly for slow-growing such as *Mycobacterium* (Botsaris et al., 2010). Different approaches have been reported, such as plaque assays, PCR/qPCR, bioluminescent reporters, among others (Alanis Villa, Griffiths, & Kropinski, 2014; Botsaris, Liapi, Kakogiannis, Dodd, & Rees, 2013; Brovko, Anany, & Griffiths, 2012; Reiman et al., 2007). These studies highlight that phages might be a promising tool for the detection of foodborne pathogens, but their application in combination with qPCR has been so far scarce. Thus, in the current study, a method which combined both, was developed and evaluated for the fast and specific detection of viable *Salmonella* Enteritidis in chicken samples.

In the evaluation of the LoD with pure phage DNA, 0.22 fg were detected, covering eight consecutive dilutions as shown in Fig. 1a. Regarding the direct analysis of phage particles, without any specific DNA extraction, it was observed that down to 10^3 pfu/mL could be reliably detected, being this value achieved covering 10 consecutive dilutions, as depicted in Fig. 1b. These data allowed to determine a qPCR amplification efficiency of 98 and 99% respectively, both with high R^2 values (0.999 and 0.995). These results are comparable to others previously reported for phage DNA detection, even though our results for the direct detection of viral particles resulted 100 times higher (Sergueev, He, Borschel, Nikolich, & Filippov, 2010). Nonetheless, these differences did not affect the final performance of the proposed PAA-qPCR assay, as discussed below.

For food analyses, a simple thermal lysis protocol was performed before qPCR detection. This step was intended to release all the phage DNA (either free or inside phage capsids), which could be inside bacterial cells that did not complete the lytic cycle. Additionally, this was expected to increase the sensitivity of the assay. It was observed that a LoD of 8.0 cfu of *S*E in 25 g was obtained in just 6 h of co-incubation of the contaminated samples with the phage solution. Considering the initial pre-enrichment step, an overall enrichment of 9 h was enough to detect bacterial concentrations below 10 cfu/25 g (higher concentrations can be detected in shorter periods of time). This is a significant time reduction compared with other methodologies that detect *Salmonella* directly but are intended for "next-day" analysis (Garrido-Maestu, Chapela, Peñaranda, & Cabado, 2015; Rodríguez-Lázaro et al., 2014). It is worth to mention that a pre-enrichment step of 3 h was included in our protocol, in order to allow the bacteria to "recover", however this step may be shortened, as reported in other methodologies, which just give 1 h for this purpose (i.e. ISO methodology for the enumeration of *Listeria monocytogenes*), thus allowing the complete protocol to be performed in 8 h (including DNA extraction and qPCR analysis). In this sense, depending on work shifts, the presented method may be implemented for self-monitoring in the food industry, allowing "same-day" detection, which represent a very interesting advantage when compared to the standard, or other rapid methods, that usually require between 20 and 48 h.

In the present study, a total of 41 samples were analyzed, with only 1 ND observed, being this associated with a sample inoculated very close to the LoD (9 cfu/25 g). This resulted in values greater than 92%, of the performance parameters evaluated, being of particular interest the k. For this parameter, a value of 0.94 was calculated, being interpreted in the range of 0.81–1.00, which corresponds to "almost complete concordance" (Altman, 1991; Anderson et al., 2011). Additionally, the AL values for "ND-PD″ and "ND + PD″ were both 1, below 3 and 6, and thus within the limits of acceptability for sensitivity as an alternative method (D'Agostino et al., 2016; ISO, 2016a, 2016b).

To confirm that the phages only infected viable *S*E, and no other microorganism, 6 samples were spiked with three different concentrations of non-viable *S*E. As expected, no positive results were obtained, thus confirming the specificity of the assay to detect only viable *S*E.

In the current study, an ISO-compatible enrichment broth, BPW, was selected. Keeping in mind that only viable *S*E are detected, and that the phage concentration added is not enough to completely eliminate all target microorganism, all positive samples are susceptible of being confirmed following standard culture techniques, such as ISO 6579 (Gianfranceschi et al., 2014; ISO, 2003). Over the development of the current method, it was observed that direct plating of BPW enrichments on XLD not always allowed to isolate typical colonies due to the high number of interfering microorganisms. Thus, if confirmation by a classic culture method is to be performed, it would be advisable to use a culture technique that allows reducing of non-target bacteria, while increasing the number of *Salmonellae*.

Future studies will focus on alternatives to provide even faster detection. In this sense, finding phages with shorter latent periods (timing of phage-induced host cell lysis) will allow to reduce the time of analysis significantly. Additionally, the replacement of the enrichment broth, BPW, for an alternative medium capable of enhancing the growth of *Salmonella* while inhibiting, or limiting, the growth of non-target interfering microorganisms will also allow a reduction in the time of analysis. This is also critical in order to assure the stability and infectivity of the phages. In this sense, careful attention must be taken in the storage conditions of the viruses, as if not done properly, may lose infectivity and so jeopardizing the final outcome of the assay. Finally, to expand the applicability of the proposed methodology, the selection of a phage cocktail, composed of *Salmonella* phages with different specificities, will be of particular interest. This phage cocktail will allow detecting *Salmonella* spp. instead of just one serovar, and at the same time will overcome the limitation of the appearance of strains resistant to the infection by one particular phage (Bai, Kim, Ryu, & Lee, 2016; Santander & Robeson, 2007).

5. Conclusions

The proposed methodology has demonstrated the capacity to detect viable *S*E, even at very low concentrations, in about 10 h including sample treatment, DNA extraction, and qPCR analysis, thus representing a significant reduction with respect to other culture-based, and molecular biology based methods. Due to the high confidence of the results obtained, this method can be suitable for the implementation on routine laboratories.

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