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Optimization of the reactional medium and a food impact study for a colorimetric *in situ Salmonella* spp. detection method

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ABSTRACT

Foodborne pathogens are still a major concern for public health authorities. In this paper, we describe the optimization of a previously reported method which combines a highly specific capture of targeted food pathogens with an intracellular staining method. The reaction medium was optimized to simultaneously allow specific enrichment of *Salmonella* and maximize the staining of the target pathogen. This *in situ* colorimetric concept was evaluated with a broad range of food samples artificially contaminated with low levels of stressed *Salmonella* to mimic natural contamination conditions. This direct detection method compared favorably to a commercially available immunoassay system (Vidas® UP *Salmonella*), for cooked meat, dry milk powder and egg products. Globally 88% agreement was obtained between the two methods with a sensitivity of 80% and a specificity of 100% for the tested method. Main discordances were obtained with food matrices having high levels of competitive Gram negative microflora. These observations show that the design of an adapted culture medium is necessary to enhance the specific *in situ* capture and revelation system.

1. Introduction

Salmonellosis continues to be a major public health problem worldwide. According to the Center for Disease Control and Prevention (CDC), 9.4 million Americans are suffering every year from food-borne illness and 10% of these infections are caused by *Salmonella* spp. (Scallan et al., 2011). This is also the case in Europe where the European Food Safety Authority (EFSA) has reported about 120 000 cases of human salmonellosis per year between 2007 and 2009 (Pires et al., 2011).

As such, it is important for food industries to verify the absence of this pathogen in their products before release on the market. The food industries have at their disposal a range of *Salmonella* screening methods including the ISO 6579: 2002 reference method which can take four to five days to obtain a result. More rapid alternative approaches using different technologies such as Polymerase Chain Reaction (PCR) (i.e. BAX® System PCR Assay for *Salmonella*, DuPont USA), Immunoassays (e.g. Vidas® UP *Salmonella*, bioMerieux France; RIDASCREEN® *Salmonella*, r-biopharm Germany) and chromogenic media (i.e. XLD®, bioMerieux France) exist. The effectiveness of these rapid detection methods differs in terms of sensitivity and specificity, and their performances often depend on the complexity of the food matrix. These methods often require several time consuming manipulations before sample analysis. We have recently reported an alternative *in situ* colorimetric detection method using a high affinity capture surface directly placed in an enrichment medium containing tetrazolium salts. Staining of the captured cells reveals the presence of the target pathogen after enrichment to a sufficient level allowing visual observation on the device (Junillon et al., 2012). Even though capture can be highly specific, reduction of the tetrazolium salt present in the medium is shared between all the bacterial cells present. In the presence of high numbers of background microflora, competition for the coloring agent may reduce the color intensity of the captured pathogens.

As a means of optimizing the reactional medium used for the detection of *Salmonella*, we investigated the development of a specific enrichment formula which could limit the growth of competitive flora and thus enhance the staining potential of target cells. In this study, we compared the previously described medium for *Salmonella* detection (Junillon et al.) (Buffered Peptone Water + 10 mg/l of vancomycin + 0.4 g/l of triphenyltetrazolium chloride) to a recently developed proprietary medium, Buffered Peptone Water + SPT selective supplement (bioMerieux, France) for a range of tetrazolium salt concentration. Optimization studies were initially conducted using various *Salmonella* strains in pure culture conditions, followed by a food impact study using low inoculums of stressed *Salmonella* in order to mimic natural contamination events.

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2. Materials and methods

2.1. Bacterial strains and inoculums

Salmonella serotype *Paratyphi A* (O-serogroup A), *Salmonella* serotype *Typhimurium* and *Salmonella* serotype *Branderburg* (B), *Salmonella* serotype *Livingstone* and *Salmonella* serotype *Mbandaka* (C1), *Salmonella* serotype *Blockley* and *Salmonella* serotype *Newport* (C2), *Salmonella* serotype *Enteritidis*, *Salmonella* serotype *Napoli* and *Salmonella* serotype *Dublin* (D1), and *Salmonella* serotype *Senftenberg* (E4) were obtained from bioMerieux bacterial strain collection (La Balme, France) and stored at −80 °C in a glycerol-containing medium. The pure cultures of microorganisms were grown on Tryptic Soy Agar (TSA) plates overnight at 37 °C. Liquid cultures were prepared in Buffered Peptone Water (BPW) and their turbidity was adjusted with a densimat® (bioMerieux) to 0.4 McFarland. Serial dilutions were performed in BPW to obtain the desired bacterial concentrations. Bacterial counts were performed in triplicate by plating 100 µl aliquots of 10^3 Colony Forming Unit/ml (cfu/ml) and 10^2 cfu/ml dilutions on TSA plates.

Stress of the *Salmonella* strains was obtained by storing a 10^8 cfu/ml suspension at −20 °C for one week. Stress was validated when difference between TSA and Xylose-Lysine-Deoxycholate (XLD, bioMerieux) plate counts was over 0.5 $\log_{10}$.

2.2. Reagents

Triphenyltetrazolium chloride (TTC) was provided by Merck (Germany) and vancomycin by Xellia Pharmaceutical (Norway). Selective *Salmonella* Phage Technology supplement (SPT) and BPW were provided by bioMerieux (Marcy l'Etoile, France). Phosphate buffered saline pH 7.2 (PBS), carbonate buffer pH 9.6 and Tris maleate with bovine serum albumin (BSA) pH 6.2 (Millipore, USA) buffer were prepared in the laboratory.

2.3. Culture medium

For the enrichment procedures, BPW supplemented with 1 ml of SPT or BPW with 10 mg/l of vancomycin was used for the growth of *Salmonella* spp. According to manufacturer's recommendations, pure culture experiments were performed with addition of 1/10 sterilized milk powder to the medium. TTC stock solution at 50 g/l was prepared in BPW and dilutions in both media were performed to obtain solutions with TTC concentrations in the range of 0.4 to 1 g/l.

2.4. In situ detection

As previously reported (Junillon et al., 2012), the *in situ* detection method uses a Solid Phase Support (SPS, Nunc® ImmunoSticks, Nunc Denmark) which is functionalized with bio-receptors using a biotin–streptavidin complex. Two kinds of bio-ligands were used: a phage tail protein Sal-BP1-biot provided by Hyglos (Germany) and three monoclonal antibodies against *Salmonella* O-serogroups (mAb-α-O-serogroup) developed internally by bioMerieux (France). Only the lower half of the surface of the ImmunoStick blades was functionalized allowing the remaining surface to be used as a control. Functionalized SPS was then placed in sample bag during the entire incubation period. Visual observation of the SPS was performed after 24 h and the intensity of the coloration was estimated on a 0 to 5 color scale as previously defined (Junillon et al., 2012).

2.5. Sample analysis

The experimental design is similar for pure culture analysis and food sample testing. Food matrices (Table 4) were purchased from a local supermarket. A food sample (25 g) was weighed into three different sterile plastic Stomacher bags (Interscience, France). Reactional medium (225 ml) was added and the sample bag homogenized for 60 s using a Bagmixer® (Interscience, France). Two of the three homogenized samples were then artificially contaminated with 50 µl of a 10^2 cfu/ml bacterial solution to obtain approximately 5 cfu per sample. Bacteria were either non-stressed (pure culture conditions and initial ground beef testing) or stressed in all other cases. Two SPS were then placed in the medium of each sample, the bag sealed and incubated at 37 °C for 24 h. After incubation, observation of the SPS was performed by tilting the bag to allow the emergence of the SPS from the solution. All experiments were performed in duplicate except for following matrices like some raw meat products (chicken, chipolata, carpaccio, bacon strip, ground beef and raw ham) which were performed in triplicate. Moreover, fresh eggs and dry milk powder were tested 4 and 5 times respectively.

2.6. Comparative analysis of the proposed method with Vidas® method

For the food impact evaluation step, a comparative analysis using Vidas® UP *Salmonella* method (bioMerieux, France) was performed. After 24 h of incubation at 37 °C, 500 µl of each sample bag use for *in situ* detection was analyzed using Vidas® platform as described in the package insert.

3. Results

Table 1 shows the results obtained in pure culture conditions with 14 different *Salmonella* strains from A to E4 O-serogroups, two reactional media: the selective SPT supplement and the BPW + vancomycin medium and various TTC concentrations from 0.4 to 1 g/l. All conditions and all the strains gave a positive result (score ≥3). Best results were obtained with both media at TTC concentrations of 0.4 to 0.8 g/l. A decrease in signal intensity until limit score (3) was observed for three strains with both media at the TTC concentrations of 1 g/l. These results appear to suggest that association of a selective medium with TTC concentrations up to 0.8 g/l is preferred for *Salmonella* detection under these conditions.

To confirm the results obtained with pure cultures, an initial test with ground beef matrix using both media and TTC concentrations of 0.4 to 0.8 g/l was performed. As shown in Table 2, mostly low scores were obtained for the BPW + vancomycin medium especially for 0.4 and 0.8 g/l of TTC. A TTC concentration of 0.6 g/l gave the best results for both media and was retained for further use. As no important differences between the selective media were observed in the results obtained, investigations were pursued using the same food matrix with low inoculums of stressed *Salmonella*. The results are summarized in Table 3 and show very lower scores with the BPW + vancomycin medium in comparison to SPT supplemented BPW.

These optimized conditions were then evaluated on a large range of different food matrices artificially contaminated with a stressed *Salmonella* strain. Table 4 shows all the food matrices tested in this study representing the most frequent food categories incriminated in *Salmonella* outbreaks with their initial contamination levels concerning total flora (TVC), *Enterobacteria* (EB) and Coliform bacteria (EC). Table 5 summarizes the results obtained with the optimized conditions previously defined compared to the Vidas® UP *Salmonella* method. Favorable results for cooked meat and dry milk food categories were obtained as all tests were correlated between both methods and no false positive or negative signals were observed. With egg products only 1 false negative result was obtained from the 19 sample tested. Compound salads and meat-based products gave 65 and 75% correlation with the Vidas® method respectively. Finally, for the 114 tests performed (80 spiked and 34 negative controls), 53 were positive with both methods and 46 samples tested were negative with both methods. No false positive was observed, and 13 false negatives were obtained with the *in situ* detection. This corresponds to 88% agreement between the new method and the Vidas® UP