Canadian Journal of Microbiology

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Journal:	<i>Canadian Journal of Microbiology</i>
Manuscript ID	cjm-2018-0547.R2
Manuscript Type:	Article
Date Submitted by the Author:	06-Dec-2018
Complete List of Authors:	Zhai, Ligong; Anhui Science and Technology University; Nanjing Agricultural University Li, Junjie; Nanjing Agricultural University Tao, Tingting; Nanjing Agricultural University Lu, Zhaoxin; Colleges of Food Science and Technology, Nanjing Agricultural University Lv, Fengxia; Nanjing Agricultural University Bie, Xiaomei; Colleges of Food Science and Technology, Nanjing Agricultural University
Keyword:	<i>S. Heidelberg</i> , real-time polymerase chain reaction, comparative genomics, PMA
Is the invited manuscript for consideration in a Special Issue? :	Not applicable (regular submission)

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Propidium monoazide (PMA) real-time PCR amplification for viable *Salmonella* species and *S. Heidelberg* in pork

Ligong Zhai ^{a,b}, Junjie Li ^a, Tingting Tao ^a, Zhaoxin Lu ^a, Fengxia Lv ^a, Xiaomei Bie ^{a,1}

a College of Food Science and Technology, Nanjing Agricultural University, Key Laboratory of Food Processing and Quality Control, Ministry of Agriculture of China

b College of Food Engineering, Anhui Science and Technology University

¹Corresponding author; e-mail: gavin340@126.com

Draft

Abstract—*Salmonella enterica* serovar Heidelberg causes foodborne infections and is a major threat to the food chain and public health. In this study, we aimed to develop a rapid molecular typing approach to identify *S. enterica* serovar Heidelberg. Using comparative genomics, four serovar-specific gene fragments were identified, and a real-time polymerase chain reaction (PCR) combined with propidium monoazide (PMA) pretreatment method was developed for simultaneous detection of viable *Salmonella* sp. (*invA*) and *S. Heidelberg*(*SeHA* C3258). The assay showed 100% specificity for all strains tested. The assay was able to effectively distinguish viable or dead cell using PMA . The detection limit was 2.4 CFU/mL following a 6-h incubation in enrichment LB medium, and the assay could detect 1.7×10^2 CFU/mL in the presence of pork background flora. In artificially contaminated pork, real-time PCR detected inoculum levels of 1.15 CFU/25 g of pork after a 6-h enrichment. Thus, our findings indicated that this comparative genomics approach could be used to screen for serovar-specific fragments and that real-time PCR with PMA was a simple and reliable method for detecting viability of *Salmonella* species and *S. Heidelberg*.

Keywords: *S. Heidelberg*, real-time polymerase chain reaction, comparative genomics, PMA

Introduction

Nontyphoidal serovars of *Salmonella* species are the causative agents of outbreaks and sporadic occurrences of systemic salmonellosis. *Salmonella enterica* serovar Heidelberg is a representative nontyphoidal serovar and a member of the *S. enterica* serogroup B subspecies (Andrysiak et al. 2008). *S. Heidelberg* is highly prevalent in North America (Hoffmann et al. 2013; Kozak et al. 2013). While most infections cause only moderate, self-limited diarrheal symptoms, more severe, invasive infections result in major complications, including meningitis, reactive arthritis, and myocarditis (Kennedy et al. 2004; Olsen 2001). Outbreaks of *S. Heidelberg* infections have been linked to consumption of contaminated food of animal origin, including turkey, chicken, and eggs, while reports of person-to-person or animal-to-person transmission are rare. *Salmonella* infection outbreaks can be caused by a single serotype (Jackson et al. 2013). Thus, rapid detection of *S. Heidelberg* would be highly beneficial for testing poultry products.

According to the Kauffmann-White classification scheme, *Salmonella* species are classified into serovars, which have a unique combinations of somatic (O), capsular (Vi), and flagellar (H1, H2) antigens (Popoff et al. 2004). Detection methods for *Salmonella* serotypes include conventional biochemical and serotyping methods (Huang et al. 1999; Kumar et al. 2008). However, enzyme-linked immunosorbent assay (ELISA) (Kumar et al. 2008), polymerase chain reaction (PCR), and real-time PCR (Gonzalez-Escalona et al. 2012) can also be used. Traditional methods are time-consuming, laborious, and costly and have been shown to result in incorrect

typing in 5–8% of cases due to the loss of surface antigens (Kim et al. 2006; Schrader et al. 2008). The ELISA method also has many disadvantages, including crossreaction with other microbes (Lee et al. 1990) and low detection limits due to the low binding capacity of the antigen-antibody complex.

Rapid detection of *Salmonella* species is vital for the food industry and for public health. Many molecular methods, including PCR and real-time PCR, have been developed for detection of pathogens (O'Regan et al. 2008; Silva et al. 2011). These molecular approaches have advantages over conventional methods in terms of specificity and sensitivity (Apfalter et al. 2003). In particular, TaqMan probes target specific genes internal to primer sites, and real-time PCR assays based on fluorescent probes are more specific than conventional PCR approaches. Numerous studies have reported the detection of prevalent *Salmonella* serovars by real-time PCR (Gonzalez-Escalona et al. 2012; Persson et al. 2012). Indeed, real-time PCR is the primary technique for serotype identification. Whole-genome *Salmonella* sequencing projects have revealed high sequence conservation, with only 10–20% sequence divergence in the genes of different serovars (Porwollik et al. 2004). Comparative genomics can identify specific *Salmonella* serovars by combining bioinformatics and PCR validation. Comparative genomics has recently identified several serotype-specific targets (*STM4495*, *SEN1392*, *SCH_0971*, *SeD_A1226*, and *SG1033*) in *S. Typhimurium*, *S. Enteritidis*, *S. Choleraesuis*, *S. Dublin*, and *S. Gallinaru*, respectively (Yuan et al. 2013).

The molecular detection is currently used to detect the contamination of

Salmonella in foods(Anejo-Okopi et al. 2016; Zhuang et al. 2014). However the methods are able to determine the viable cells and dead cells' DNA at the same time. Propidium monoazide (PMA) as a more selective agent has been used to overcome this problem of discrimination viable and non-viable cells(McLellan et al. 2016). Theoretically, the reagent is only able to penetrate the damaged membrane of dead cells and attach to DNA after a light exposure, interfering with DNA amplification(Taylor et al. 2014). Until now, the combination of PCR or RT-PCR with the PMA sample treatment step has been applied to differentiate the viability of food-borne pathogens in food samples(Kim et al. 2014; Kim and Wuertz 2015; Pacholewicz et al. 2013).

Accordingly, in this study, we aimed to evaluate the use of a comparative genomics approach for identification of unique genes for the detection of *S. Heidelberg*. We developed a real-time PCR assay combined with the treatment of PMA that targeted *invA* (Gonzalez-Escalona et al. 2009) and *SeHA_C3258* to facilitate detection the viability of *Salmonella* species, particularly *S. Heidelberg*.

Materials and methods

Bacterial strains

A total of 53 *Salmonella* strains representing 34 different serovars and an additional 18 non-*Salmonella* foodborne pathogens (Table 1) were acquired from the China Center of Industrial Culture Collection (CICC), the National Center for Medical Culture Collections (CMCC), Guangdong Culture Collections (GIMCC), the

American Type Culture Collection (ATCC), and Eastern Regional Research Center.

Strains were stored at -70°C in Luria-Bertani (LB) medium containing 25% glycerol.

Five *S. Heidelberg* strains, i.e., BEAR097339, BEAR097801, BEAR097391, BEAR097397, and BEAR097807, were included.

Screening for *S. Heidelberg*-specific genes

The genomic sequence of *S. Heidelberg* strain SL476 (NC_011083.1) was downloaded from the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/>). To identify *S. Heidelberg*-specific genes, 5,009 annotated protein-coding sequences of *S. Heidelberg* strain SL476 were screened for nucleotide sequence similarity against fragments or genomes from *Salmonella* and non-*Salmonella* species available at NCBI using BLASTN. Sequences exhibiting low homology with non-*Salmonella* species and high homology with all *S. Heidelberg* sequences were considered specific targets for *S. Heidelberg* detection and were used as candidate detection fragments for PCR. Serovar-specific genes are listed in Table 2.

PMA pretreatment

PMA (Biotium, Inc, Hayward, CA) was prepared in 20% DMSO to obtain a 10 mM stock solution and stored at -20 °C in the dark. The 10 µL of PMA stock solution was transferred to 1 mL of culture sample to achieve a final working concentration of 100 µM. Following, the samples were mixed well by vortexing and incubated 5 min at room temperature in the dark. After the samples placed on ice were exposed to a 500-W halogen light for 10 min at a distance of 20 cm and ice was replenished every

5 min to avoid excessive heating.

Genomic DNA extraction

Bacterial genomic DNA was isolated from enrichment cultures and PMA treatment using a bacterial genomic DNA extraction kit (Sangon Biotech China) and an E.N.Z.A bacteria genome kit (Omega, USA) according to the manufacturer's instructions. Extracted DNA was validated by PCR using primer 139-141 and stored at -20°C until use. DNA concentration was measured using a NanoDrop 2000 UV spectrophotometer (Thermo Scientific, MA, USA).

Real-time PCR assay for detection of *Salmonella* species and *S. Heidelberg*

Real-time PCR was used to amplify *Salmonella* and *S. Heidelberg* marker genes using two primer pairs (forward and reverse). Each 20-μL reaction contained 10 μL of 2× Quantitect PCR mixture, 0.8 μL pHAT2 primer, 0.4 μL SH2-t probe, 0.4 μL invA_176F and invA_291R primers, 0.4 μL invA_Tx_208 probe, and 2 μL template DNA (Gonzalez-Escalona et al. 2009). The PCR program consisted of an initial denaturation at 94°C for 4 min, followed by 40 amplification cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Fluorescence signals based on FAM, JOE, and ROX were detected during each elongation step, and the signal was considered positive when the threshold cycle (Ct) value was less than 35.

Effectiveness of real-time PCR

Purified genomic DNA from *S. Heidelberg* was serially diluted 10-fold with deionized water from 112.4 ng/μL to 1.124 pg/μL and subjected to real-time PCR amplification. A 1-μL aliquot of each dilution was used as template. The resulting standard curves

were used to evaluate the linear range and calculate the real-time PCR efficiencies by determination of the R^2 values.

The effect of PMA-real-time PCR on *S. Heidelberg*

S. Heidelberg was grown overnight at 37 °C and then serially diluted 100-fold with sterile saline to obtain different suspension of : i) 7.62×10^7 CFU/mL viable cells and dead cells (10 min at 95 °C), ii) 7.62×10^5 CFU/mL viable cells and dead cells (10 min at 95 °C) and iii) 7.62×10^3 CFU/mL viable cells and dead cells (10 min at 95 °C). After, each sample was treated separately by PMA (100 μ M) and without PMA, then DNA was extracted for real-time PCR. The all experiments were performed in triplicate.

Determination of the limit of detection

S. Heidelberg was cultured overnight and then serially diluted in sterile saline in 10-fold steps down to 1:10⁷. One milliliter of each dilution was inoculated into an equal volume of fresh LB medium and incubated at 37°C for 6h. After enrichment, 1 mL of each dilution was treated by PMA (final concentration of 100 μ M) and used for DNA extraction with an E.N.Z.A bacteria genome kit (Tatavarthy and Cannons, 2010). Templates were tested by real-time PCR in triplicate.

Detection of *S. Heidelberg* in the presence of background flora

To investigate the robustness of the assay without selective enrichment, the method was tested in the presence of a complex background microflora from pork. An overnight culture of *S. Heidelberg* CICC21487 was diluted 10-fold using buffered

peptone water, and the pork sample was confirmed to be *Salmonella*-free by standard microbiological methods. Ten grams of pork was inoculated into 90 mL LB at 37°C for 12 h, and 500- μ L aliquots were collected and combined with 500 μ L of the pork background flora culture. Mixtures were treated by PMA (final concentration of 100 μ M) and subjected to nucleic acid extraction for testing to real-time PCR assay.

Artificial contamination of pork samples

S. Heidelberg was grown in LB at 37°C overnight and serially diluted 10-fold with sterile saline solution. Pork samples from the local supermarket were determined to be free of *S. Heidelberg* by standard methods (GB4789.4-2008, www.bzxz.net/bzxz/114499.html). Samples (25 g) were inoculated with *S. Heidelberg* in the range of 1 to 10² CFU/25 g of pork. After contamination, samples were transferred to LB medium and incubated at 37°C for 4, 6, 8, or 10 h. Each sample was treated by PMA and then DNA was extracted using the boiling method (Tatavarthy and Cannons, 2010). PMA-real-time PCR was considered positive when the Ct value was less than 35. The CFU in the initial inoculum was determined using standard cell counting methods.

Results

Comparative genomics for mining *S. Heidelberg*-specific target genes

The genomic sequences of *S. Heidelberg* were compared with genomic sequences in the NCBI database using BLASTN. Two genomic fragments were present only in *S. Heidelberg*, and four gene sequences were present only in *S. Heidelberg* and

pEcWSU1 from *Enterobacter cloacae* (data not shown). Based on the above candidate targets, nine primer sets were designed using Primer Premier 5.0 for specific amplification of the fragments. Of these, only six primer sets exhibited 100% inclusivity for six *S. Heidelberg* strains and 100% exclusivity for 65 non-*S. Heidelberg* strains (including 53 *Salmonella* strains and 18 non-*Salmonella* strains). The six pairs of primers were specific for all four different genomic fragments (Table 2).

Real-time PCR detection of *S. Heidelberg*

A real-time PCR assay targeting *SeHA_C3258* and *invA* was further developed for the detection of *Salmonella* spp. and *S. Heidelberg*. The Ct value of FAM and JOE dyes was 35 cycles (with water as a negative control). Ct values for *S. Heidelberg*-specific JOE (*SeHA_C3259*) and the *Salmonella* spp.-specific FAM (*invA*) were less than 35 cycles (Fig. 1), and Ct values greater than 35 cycles were deemed negative. If only the FAM Ct value was below 35 cycles, the sample was considered to contain *Salmonella* spp., and the serotype should be verified by additional tests. Correlation coefficients (R^2) of standard curves based on *SeHA_C3258* and *invA* were determined to be 0.996 and 0.995 (Fig. 2). The assay was therefore considered suitable for qualitative but not quantitative purposes.

Effectiveness of PMA treatment to differentiate live and dead *S. Heidelberg*

The effect of PMA pretreatment was evaluated by discriminate of selective concentration of live and dead *S. Heidelberg* cells (Table 4). Some general trends could be depicted, concerning the effect of PMA treatment. The PMA treatment did

not affect the mean cycle threshold of viable cells when the PMA final concentration was 100 μ M. There was a noticeable difference between the result with PMA pretreatment and without PMA for detection of heat-killed cells by the real-time PCR. Therefore, this study used a PMA treatment to discriminate live-dead cells of *S. Heidelberg*.

Specificity and sensitivity

The specificity of the PMA-real-time PCR assay was evaluated using 53 *Salmonella* and 18 non-*Salmonella* strains (Table 1). For multiplex and real-time PCR, primers based on the *invA* gene were verified to be 100% inclusive for *Salmonella* spp., and there was no amplification of non-*Salmonella* species. Likewise, the serotype Heidelberg-specific primers did not amplify any non-*S. Heidelberg* strains.

Regarding sensitivity, 2.4 CFU/mL of sample yielded a measureable fluorescence signal in the JOE and FAM dye channels after a 10-h enrichment step, and Ct values were 30.81 ± 0.29 and 31.76 ± 0.53 , respectively (Table 5). When an enrichment step was not included, the sensitivity was only 2.4×10^4 CFU/mL, and the Ct values of the sample and negative control were similar ($\sim 10^{-1}$ CFU/mL). The detection limit of the assay was therefore 2.4 CFU/mL.

Detection of *S. Heidelberg* in the presence of background flora

The detection limit of the PMA- real-time PCR assay in the presence of background flora was evaluated, and the total aerobic plate count from a 12-h enrichment of the pork sample was 1.16×10^8 CFU/mL. The sensitivity in the presence of pork background flora was also tested, and the detection limit was found to be $\sim 1.7 \times 10^2$

CFU/mL (Table 6). Under these conditions, the assay could not yield a satisfactory amplification curve at 17 CFU/mL (Table 6).

Detection of *Salmonella* in artificially contaminated pork

Pork samples were spiked with 1 to 10^2 CFU/10 mL of *S. Heidelberg*, and the detection method was performed after 4, 6, 8, and 10 h of enrichment. *S. Heidelberg* was detected at 100% after a 6-h enrichment, with FAM Ct values (*Salmonella* spp.) ranging from 24.326 ± 0.209 to 28.659 ± 0.375 and JOE Ct values (*S. Heidelberg*) ranging from 26.387 ± 0.041 to 30.545 ± 0.003 . After a 4-h enrichment, FAM and JOE Ct values in these samples were 32.498 ± 0.36 and 34.064 ± 0.27 , respectively (10^2 CFU *S. Heidelberg* per 25 g pork); thus, these results were considered positive (Fig. 3).

Discussion

Identification of *S. Heidelberg*-specific target genes

In previous reports, detection of *S. Heidelberg* was mainly accomplished using traditional microbiological methods (Galanis and Parmley 2012; Schrader et al. 2008), which require 5–7 days. Serovars are usually identified based on antigenic differences in lipopolysaccharide (LPS) and flagellin (H1 and H2) antigens. A recent report characterized the genes for O-antigen biosynthesis and the central variable region of the H1 and H2 flagellin gene in *Salmonella* to identify O- and H-specific alleles (Yang et al. 2012), and PCR primers were developed for the identification of *Salmonella* serogroups A, B, C1, and C2 and of the serovar *S. Heidelberg* (Hong et al.

2008). Such allelotyping PCR methodologies can simultaneously detect multiple different serotypes; however, the detection process requires three different multiplex PCRs for O, H1 and H2 alleles, as well as a separate electrophoresis step. While this approach provides faster results than traditional microbiological methods, it is not ideal for use in the food industry due to the complex detection and analytical processes (Maurer et al. 2011).

In order to detect *S. Heidelberg* in poultry directly, we developed a duplex real-time PCR method targeting serovar-specific genes by utilizing a comparative genomics method. With the increasing availability of whole-genome sequences, it is possible to make use of bioinformatics tools to identify serovar-specific target genes to detect serovars Typhi, Typhimurium, and Enteritidis (Charlton et al. 2005; Park et al. 2009). Serovar-specific gene mining allows the detection of unique target sequences for which a function has yet to be assigned. In this study, we first identified four *S. Heidelberg*-specific genomic sequences based on the results of a BLASTN search. The functions of the four identified genes were listed in the NCBI database (Table 2). Primer sets targeting the four genes (*SeHA_C2639*, *SeHA_C2640*, *SeHA_C3258*, and *SeHA_C3259*) were further tested against 53 *Salmonella* and 18 non-*Salmonella* strains, most of which are known to be associated with food.

Detection of *S. Heidelberg*

Only detection of viable pathogenic bacteria could pose potential threats in food microbiology. However, PCR based-DNA detection is difficult to distinguish between live and dead bacteria (Gyawali et al. 2016). In our work, PMA was applied for

solving the problem. PMA could penetrate dead cells and binds covalently to DNA to block PCR amplification (Reyneke et al. 2017). In contrast, PMA cannot penetrate viable cells therefore PMA-combined real-time PCR assay could detect viable bacteria on food sample. According to present report, the PMA-PCR protocol was able to distinguish viable and dead cells when a concentration of PMA pretreatment was 100 μ M (Duarte et al. 2015). In our work, the PMA concentration was effective. For development of the real-time PCR assay, a pHAT2 primer was designed for detecting serotype Heidelberg and *Salmonella* spp. (Gonzalez-Escalona et al. 2009). Primers targeting *invA* were important for enhancing detection specificity since they could distinguish between *Salmonella* and non-*Salmonella* species, even in the complex microbial environments found in food. The *invA* gene has previously been used to detect *Salmonella* species by PCR-based assays performed in Europe (Hong et al. 2008). Thus, we included this gene as an internal amplification control (IAC) in the real-time PCR assay developed in the present study. The specificity of both PCR procedures was similar to the allelotyping PCR method for detection of *S. Heidelberg*, as described previously (Hong et al. 2008). This previous study utilized the divergence of two glycosyl synthase genes responsible for the specific O-antigen of *Salmonella* species, along with allele-specific sequences within the hypervariable regions of the H1 and H2 antigens, to develop four multiplex PCR assays that could detect four different *Salmonella* serovars (Hong et al. 2008). The detection system was able to identify not only *S. Heidelberg*, but also *S. enterica* serovars Enteritidis, Hadar, and Typhimurium. However, this method of detection requires four PCR

assays and an electrophoresis step, which together are highly time consuming. In the present study, the identification of *S. Heidelberg* required only one real-time PCR assay, and the results could be summarized with a single Ct value, indicating that this method was simpler and faster. An additional advantage of our real-time PCR assay is the identification of *Salmonella* species and *S. Heidelberg* serotype in particular in one rapid and reliable step. PCR-based approaches have been successfully applied to identify various serovars, including Enteritidis, Typhimurium, Choleraesuis, Hadar, Paratyphi A, and Paratyphi B. However, our study is the first to report the detection of *S. Heidelberg* using PMA-real-time PCR rather than allelotyping PCR and multiplex PCR.

Enrichment and sensitivity

In this study, the detection method included a nonselective enrichment step and a DNA extraction step. The aim of the enrichment step was to increase the number of viable cells (O'Regan et al. 2008), which effectively diluted inhibitory substances such as fats, glycogen, calcium salts, proteases, and phenolic compounds prior to the PCR step (Silva et al. 2011). The real-time PCR assay was able to detect *S. Heidelberg* in cultures inoculated at levels of 2.4 CFU/mL, with FAM and JOE dye Ct values of 31.76 and 30.81, respectively. Compared with a previously published real-time assay targeting *S. Enteritidis*, the duplex real-time PCR assay has a higher detection limit (Malorny et al. 2007). In the present study, sensitivity was superior after the enrichment step, consistent with a previous study reporting improved PCR-based detection of *Salmonella* in poultry samples following enrichment

(O'Regan et al. 2008). We therefore concluded that pre-enrichment was an essential step for the reliable detection of *S. Heidelberg*. We also investigated the ability of the assay to detect *S. Heidelberg* in the presence of a complex pork background flora, and *S. Heidelberg* was detected at levels as low as 1.7×10^2 CFU/mL under these conditions. This is a significant improvement to the sensitivity of 1.3×10^3 CFU/mL reported in a previous real-time PCR study of *Salmonella* detection (Chen et al. 2010).

Detection of artificially contaminated pork

The ability of the real-time PCR assay to detect *S. Heidelberg* in artificially contaminated pork was investigated, and *S. Heidelberg* was positively identified in spiked samples at levels as low as 1 CFU/25 g pork after a 6-h enrichment step. The complete detection process therefore only required 10 h, which was significantly shorter than a previous multiplex PCR method involving electrophoresis (Hong et al. 2008). In summary, the use of Taqman probes for the real-time PCR assay had several advantages over multiplex PCR approaches, including greater sensitivity, easier analysis, and shorter overall detection time.

CONCLUSIONS

In this study, we report novel targets for the identification of *S. Heidelberg* using a real-time PCR combined PMA pretreatment method based on comparative genomics. This approach successfully screened for *Salmonella* species and differentiated *S. enterica* serovar Heidelberg and identified viable cells. Accordingly, this method may

be applicable for the rapid identification of *Salmonella* serotype in routine diagnostic laboratories.

ACKNOWLEDGEMENTS

Funding: This work was funded by grants from the Anhui Science and Technology University Talent Introduction Project (grant number SPYJ201602), Jiangsu Agricultural Science and Technology Independent innovation Foundation (grant no.CX(18)3053) and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

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Table Headers

Table 1. *Salmonella* and non-*Salmonella* strains used in this study

+ = positive result

- = negative result

*¹ = provided by P M. Fratamico from eastern regional research center

*² = laboratory-stored strain

Table 2. Serovar-specific genes in *S. Heidelberg*.

Table 3. Primer and probe sets used in real-time PCR combined PMA detection of *S. Heidelberg*

Table 4. Real-time PCR combined PMA method for detection of viable and dead *S. Heidelberg* (CICC21487)

ND =without amplification curve

a =Each condition was replicated three times, and Ct values are means ± standard deviations

b =Live and dead cells were treated with 100 µM PMA (Final conclusion)

Table 5. Sensitivity of real-time PCR combined PMA for detection of *S. Heidelberg* (CICC21487)

+ = positive result

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ND = without amplification curve

Table 6. Detection of *S. Heidelberg* (CICC21487) in the presence of pork background flora

+ = positive result

- = negative result

Figure Captions

Fig. 1. Detection of *S. Heidelberg* (CICC21487) by real-time PCR combined PMA through the amplification of *SeHA_C3258* and *invA*. CN = negative control in which water was used as template.

Fig. 2. Standard curves of real-time PCR combined PMA assays based on the amplification of *invA* (A) and *SeHA_C3258* (B) genes. Each data point represents the mean Ct value obtained from 112.4 ng/μL to 1.124 pg/μL of DNA.

Fig. 3. Detection of viable *S. Heidelberg* cells in artificially contaminated pork samples using the real-time PCR combined with PMA.

The Ct values were calculated to quantify amplification of *SeHA_C3258* from JOE (A) and *invA* from FAM (B)

Fig. 1. Detection of *S. Heidelberg* (CICC21487) by real-time PCR combined PMA through the amplification of *SeHA_C3258* and *invA*. CN = negative control in which water was used as template.

131x104mm (300 x 300 DPI)

Fig. 2. Standard curves of real-time PCR combined PMA assays based on the amplification of *invA* (A) and *SeHA_C3258* (B) genes. Each data point represents the mean Ct value obtained from 112.4 ng/ μ L to 1.124 pg/ μ L of DNA.

140x150mm (300 x 300 DPI)

Fig. 3. Detection of viable *S. Heidelberg* cells in artificially contaminated pork samples using the real-time PCR combined with PMA. The Ct values were calculated to quantify amplification of *SeHA_C3258* from JOE (A) and *invA* from FAM (B)

299x420mm (300 x 300 DPI)

TABLES

Table 1. *Salmonella* and non-*Salmonella* strains used in this study

Strain (<i>Salmonella</i>)	Source	Number	Result	Strain (<i>Salmonella</i>)	Source	Number	Result	Strain (non- <i>Salmonella</i>)	Number	Source	Result
<i>S. Paratyphi</i> A	CMCC50001	1	—	<i>S. Kentucky</i>	CICC21488	1	—	<i>Escherichia coli</i>	1	ATCC35150	—
<i>S. Paratyphi</i> A	CICC21501	1	—	<i>S. Bazenheid</i>	CICC21587	1	—	<i>Escherichia coli</i>	1	ATCC43889	—
<i>S. Saintpaul</i>	CICC21486	1	—	<i>S. Thphi</i>	CMCC50071	1	—	<i>Enterococcus faecalis</i>	1	ATCC12953	—
<i>S. Paratyphi</i> B	CICC21495	1	—	<i>S. Enteritidis</i>	CICC21527	1	—	<i>Enterococcus faecalis</i>	1	ATCC29212	—
<i>S. Heidelberg</i>	CICC21487	1	+	<i>S. Enteritidis</i>	CICC21482	1	—	<i>Enterococcus avium</i>	1	ATCC14025	—
<i>S. Heidelberg</i> * ¹		5	+	<i>S. Enteritidis</i>	CVCC3374	1	—	<i>Klebsiella pneumoniae</i>	1	ATCC13884	—
<i>S. Typhimurium</i>	CMCC51005	1	—	<i>S. Enteritidis</i>	CMCC50041	1	—	<i>Staphylococcus aureus</i>	1	ATCC29213	—
<i>S. Typhimurium</i>	CICC21483	1	—	<i>S. Enteritidis</i>	CMCC50071	1	—	<i>Staphylococcus aureus</i>	1	ATCC25923	—
<i>S. Typhimurium</i>	CVCC3384	1	—	<i>S. Enteritidis</i> * ²		2	—	<i>Serratia marcescens</i>	1	CICC10187	—
<i>S. Typhimurium</i> * ²		3	—	<i>S. Dublin</i>	CICC21497	1	—	<i>Bacillus pumilus</i>	1	CMCC63202	—
<i>S. Bredeney</i> * ²		1	—	<i>S. Dublin</i>	CMCC50761	1	—	<i>Bacillus cereus</i> * ²	1		—
<i>S. Derby</i> * ²		1	—	<i>S. Miami</i>	CICC21509	1	—	<i>Pseudomonas fluorescens</i> * ²	1		—
<i>S. Paratyphi</i> C	CICC21512	1	—	<i>S. Eastbourne</i>	CICC21508	1	—	<i>Listeria grayi</i>	1	CICC21670	—
<i>S. Montevideo</i>	CICC21588	1	—	<i>S. Anatum</i>	CICC21498	1	—	<i>Listeria seeligeri</i>	1	CICC21671	—
<i>S. Jerusalem</i>	CICC21651	1	—	<i>S. Mleagridis</i>	CICC21511	1	—	<i>Listeria welshimeri</i>	1	CICC21672	—
<i>S. Bonn</i>	CICC21677	1	—	<i>S. London</i> * ²		1	—	<i>Listeria monocytogenes</i>	1	CICC21662	—
<i>S. Choleraesuis</i>	CICC21493	1	—	<i>S. Senftenberg</i>	CICC21502	1	—	<i>Listeria ivanovii</i>	1	CICC21663	—
<i>S. Choleraesuis</i>	ATCC13312	1	—	<i>S. Aberdeen</i>	CICC21492	1	—	<i>Listeria innocua</i>	1	CICC10417	—

<i>S. Thompson</i>	<i>CICC21480</i>	1	—	<i>S. Blockley</i>	<i>CICC21489</i>	1	—
<i>S. Potsdam</i>	<i>CICC21500</i>	1	—	<i>S. Adelaide</i>	<i>CICC21505</i>	1	—
<i>S. Braenderup</i>	<i>ATCC19812</i>	1	—	<i>S. Wandsworth</i>	<i>CICC21504</i>	1	—
<i>S. Bonariensis</i>	<i>CICC21496</i>	1	—	<i>S. Dakar</i>	<i>CICC21507</i>	1	—
<i>S. Bovismorbificans</i>	<i>CICC21499</i>	1	—	<i>S. Agona</i>	<i>CICC21586</i>	1	—

+ = positive result

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*² = laboratory-stored strain

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Table 2. Serovar-specific genes in *S. Heidelberg*.

Serovar	Source	Serovar-specific gene	Annotation
<i>S. Heidelberg</i>	Genome	<i>SeHA_C2639</i>	Abortive phage infection protein AIPR
		<i>SeHA_C2640</i>	Arylsulfatase regulator
		<i>SeHA_C3258</i>	DeoR family transcriptional regulator
		<i>SeHA_C3259</i>	Ribose-5-phosphate isomerase B

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Table 3. Primer and probe sets used in real-time PCR combined PMA detection of *S. Heidelberg*

Target organism	Gene	Primer	sequence ((5'–3'))	Reference
<i>S. Heidelberg</i>	<i>SeHA_C3258</i>	pHAT2-f	TATCCCCCCAATAGCTCAAT	This study
		pHAT2-r	CCCACCCACCAAAGACAGAAG	
		SH2-t	JOE-TGACTCGATCAGCACACTGGCCACCGG-TAMRA	
<i>Salmonella</i>	<i>invA</i>	invA_176F	CAACGTTTCCTGCGGTACTGT	Gonzalez N et al. (2009)
		invA_291R	CCCGAACGTGGCGATAATT	
		invA_Tx_208	FAM-CTCTTTCGTCTGGCATTATCGATCAGTACCA-TAMRA	

Table 4. Real-time PCR combined PMA method for detection of viable and dead *S. Heidelberg* (CICC21487)

<i>S. Heidelberg</i> (CFU/mL)	Duplex real-time PCR							
	<i>invA</i> Ct value ^a				<i>SeHA_C3258</i> Ct value ^a			
	Live cells		Dead cells		Live cells		Dead cells	
	-PMA ^b	+PMA	-PMA	+PMA	-PMA	+PMA	-PMA	+PMA
0	35.48 ± 0.62	36.76 ± 0.43	35.94 ± 0.67	ND	36.78 ± 0.92	36.82 ± 0.81	35.14 ± 0.88	ND
7.62×10 ²	33.76 ± 0.53	34.81 ± 0.29	34.76 ± 0.87	ND	33.43 ± 0.63	34.42 ± 0.23	34.45 ± 0.79	ND
7.62×10 ⁵	19.36 ± 0.21	18.73 ± 0.69	18.23 ± 0.69	ND	20.12 ± 0.11	19.89 ± 0.46	20.43 ± 0.72	ND
7.62×10 ⁸	14.71 ± 0.18	13.21 ± 0.26	12.15 ± 0.43	ND	15.84 ± 0.38	14.47 ± 0.56	15.97 ± 0.63	ND

ND =without amplification curve

a =Each condition was replicated three times, and Ct values are means ± standard deviations

b =Live and dead cells were treated with 100 µM PMA (Final conclusion)

Table 5. Sensitivity of real-time PCR combined PMA for detection of *S. Heidelberg* (CICC21487)

<i>S. Heidelberg</i> (CFU/ml)	0h			6h		
	<i>invA</i>	<i>SeHA_C3258</i>	Result	<i>invA</i>	<i>SeHA_C3258</i>	Result
2.4×10^7	22.32 ± 0.39	20.41 ± 0.29	+	16.28 ± 0.50	15.07 ± 0.41	+
2.4×10^6	24.35 ± 0.28	24.02 ± 0.27	+	16.29 ± 0.56	15.26 ± 0.54	+
2.4×10^5	29.21 ± 0.51	30.13 ± 0.29	+	15.98 ± 0.17	14.15 ± 0.43	+
2.4×10^4	31.35 ± 0.28	30.02 ± 0.27	+	15.59 ± 0.20	13.76 ± 0.73	+
2.4×10^3	32.21 ± 0.51	31.13 ± 0.29	+	15.47 ± 0.26	13.75 ± 0.46	+
2.4×10^2	34.82 ± 0.37	34.46 ± 0.26	+	18.89 ± 0.49	17.64 ± 0.57	+
2.4×10^1	35.28 ± 1.46	35.03 ± 1.71	—	21.87 ± 0.39	20.37 ± 0.38	+
2.4×10^0	ND	ND	—	31.76 ± 0.53	30.81 ± 0.29	+
2.4×10^{-1}	ND	ND	—	35.36 ± 0.41	34.01 ± 0.35	—

+ = positive result

- = negative result

ND = without amplification curve

Table 6. Detection of *S. Heidelberg* (CICC21487) in the presence of pork background flora

<i>S. Heidelberg</i> (CFU/ml)	<i>invA</i> (Ct ± SD) (n = 3)	<i>SeHA_C3258</i> (Ct ± SD) (n = 3)	Result
1.7×10^7	21.97 ± 0.51	20.43 ± 0.14	+
1.7×10^6	23.91 ± 0.11	22.59 ± 0.18	+
1.7×10^5	26.39 ± 0.44	25.25 ± 0.25	+
1.7×10^4	30.48 ± 0.26	29.08 ± 0.13	+
1.7×10^3	33.64 ± 0.19	32.26 ± 0.45	+
1.7×10^2	34.31 ± 0.53	32.96 ± 0.17	+
1.7×10^1	35.64 ± 1.21	34.79 ± 1.08	—
0	35.56 ± 2.31	36.83 ± 1.78	—

+ = positive result

- = negative result