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Direct PCR – a rapid method for multiplexed detection of different serotypes of *Salmonella* in enriched pork meat samples

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Abstract

Salmonellosis, an infectious disease caused by *Salmonella* spp., is one of the most common foodborne diseases. Isolation and identification of *Salmonella* by conventional bacterial culture method is time consuming. In response to the demand for rapid on line or at site detection of pathogens, in this study, we developed a multiplex Direct PCR method for rapid detection of different *Salmonella* serotypes directly from pork meat samples without any DNA purification steps. An inhibitor-resistant Phusion *Pfu* DNA polymerase was used to overcome PCR inhibition. Four

pairs of primers including a pair of newly designed primers targeting *Salmonella* spp. at subtype level were incorporated in the multiplex Direct PCR. To maximize the efficiency of the Direct PCR, the ratio between sample and dilution buffer was optimized. The sensitivity and specificity of the multiplex Direct PCR were tested using naturally contaminated pork meat samples for detecting and subtyping of *Salmonella* spp. Conventional bacterial culture methods were used as reference to evaluate the performance of the multiplex Direct PCR. Relative accuracy, sensitivity and specificity of 98.8%; 97.6% and 100%, respectively, were achieved by the method. Application of the multiplex Direct PCR to detect *Salmonella* in pork meat at slaughter reduces the time of detection from 5-6 days by conventional bacterial culture and serotyping methods to 14 hours (including 12 hours enrichment time). Furthermore, the method poses a possibility of miniaturization and integration into a point-of-need Lab-on-a-chip system for rapid online pathogen detection.

1. Introduction

Animal food production plays an important role in the economies of the European Union (EU). In particular, Denmark is one of the top EU countries to export meat. The Danish annual production of pork is around 1,896 million kilograms. Beef and veal production reached a total of 139 million kilograms while poultry production increased up to 191 million kilograms in 2013. In Denmark, the annual budget of exported meat and meat products is estimated around 156 billion Danish krone [1]. The increasingly stringent legislation [2] together with heightened public awareness has urged the food industry and the legislation bodies to intensively test animal originated food products to ensure food safety. *Salmonella* is one of the most common foodborne pathogens found in animal food products. The *Salmonella* genus is a member of the family *Enterobacteriaceae*, and has more than 2600 serotypes [3] . In the EU, three *Salmonella* serovars: *Salmonella* Enteritidis, *Salmonella* Typhimurium and *Salmonella* Infantis are identified as the most frequently found and widely distributed *Salmonella* serovars in animals used for food production [4]. In Denmark, *Salmonella*

Typhimurium and *Salmonella* Derby are identified as the common serotypes associated with pig herds, while *Salmonella* Enteritidis is associated with layer flocks, and *Salmonella* Typhimurium and *Salmonella* Infantis are associated with broiler flocks [5]. Since *Salmonella* contaminated food products can cause severe infection, a single event of a foodborne disease outbreak can bring unimaginable economic losses. The outbreak not only poses a threat to human health [6], but also severely damages the international reputation of a product or a food company and the potential impact can be devastating [7]. Today, animal food production and distribution networks are becoming quicker and have a greater capacity than ever before. Rapid and multiplex methods suitable for online or at site screening of animal food products are urgently needed in order to expedite the timely release of products for retail distribution as well as to take necessary action to mitigate foodborne diseases outbreak and economic loss.

Up to date, conventional bacterial culture methods have been the reference methods for detection of *Salmonella* in food. The cost of bacterial culture method in Denmark including labour cost was around 50 USD per sample. The methods are expensive and time consuming, requiring up to 5 days to obtain the confirmed results [8]. Apparently, the methods were not fast enough to keep up with the pace of animal food production. In the last decades, several PCR-based methods that include PCR [9], PCR in combination with hybridization [10], immuno-PCR by combining monoclonal antibody coated magnetic bead with PCR [11], PCR in combination with conventional culture and serotyping [12] and real-time PCR [13-18] for rapid detection of *Salmonella* have been reported. However, all of these methods require a pre-enrichment step of 6-28h followed by tedious DNA isolation and purification procedures to overcome PCR inhibitors. Moreover, most of the methods can only detect certain serovar of *Salmonella*.

In order to reduce the time and complexity of detection, Direct PCR methods have been described in literature. These methods aim to use raw sample as the template for PCR amplification, thus

eliminate the need for DNA purification. The main obstacle in the developing of this Direct PCR is PCR inhibitors. Different strategies have been applied to overcome the effect of the PCR inhibitors such as addition of BSA, protease inhibitors, magnesium ions and chelation of calcium ions. However, the application of such methods depends on the type of inhibitors or specific type of samples [19]. Alternative research focus on re-engineering the thermostable polymerases either by induced point mutations or genetic recombination to enhance processivity and fidelity of the polymerase enzymes. Kermekchiev et al. (2009) reported a N-terminal deletion of mutant *Klentaq 1* - variant of *Taq* DNA polymerase that exhibits 10-100 fold higher inhibition resistant to whole blood in comparison to full-length wild type *Taq* DNA polymerase which is strongly inhibited by 0.1-1% of blood. It has been shown that mutations at codon 708 both in the *Klentaq 1* and *Taq* polymerase enhanced resistance to various PCR inhibitors such as whole blood, plasma, hemoglobin, lactoferrin, humic acid and a high concentration of intercalating dyes [20]. Wang et al. [21] reported an engineered *Pfu* DNA polymerase that has been fusion with a double-strand DNA-binding domain (Sso7d) with high robustness. The enzyme was successfully produced with good processivity, as well as high catalytic activity and enzyme stability [21-22]. *In vitro* studies have been showed that Sso7d protein can assist in annealing of complementary DNA strands [23] and can causes negative supercoiling [24]. In addition, the fusion of the Sso7d protein domain in a *Pfu* DNA polymerase showed increase in the tolerance to high salt concentration [21]. The feasibility of this reengineered DNA polymerase has been recently demonstrated by performing Direct PCR on forensic samples [25]. However, this DNA polymerase has seldom been studied or applied for detecting pathogens in food and animal samples at slaughter.

In response to the demand for fast at-site detection of pathogens, in this study, we developed a new multiplex Direct PCR to rapidly identify different *Salmonella* spp, directly from BPW enriched pork meat samples without DNA isolation and purification. This PCR assay can target *S.*

Typhimurium and *S.* Dublin in natural contaminated samples with accuracy 88.9 % (24 out of 27) and 66.7% (2 out of 3). The commonly found *Salmonella* serotype in this study is correlated with the survey report in Denmark [5]. The Direct PCR method possesses high potential for integrating into online detection systems such as Lab-on-a-chip devices, so that animal food industry and regulatory bodies can monitor food quality and food safety at much reduced time and cost.

2. Materials and Methods

2.1. Chemicals

All chemicals and reagents used in this study were of analytical grade and purchased from Pierce Inc., USA or Sigma-Aldrich, USA unless otherwise specified.

2.2. Bacterial strains and culture conditions

A total of 31 bacterial strains including 15 *Salmonella* serotypes and 16 non-*Salmonella* bacterial strains isolated from chickens or pigs were employed to study the sensitivity and the specificity of the Direct PCR (Table 1). All of the strains were provided by National Food Institute (DTU-Food). They were originated from the Culture Collection at University of Gothenburg (CCUG) Sweden, the National Collection of Type Culture (NCTC) or the Veterinary Diagnostic Laboratory (VDL) UK.

The *Salmonella* strains were resuscitated and grown on 5% Blood agar (BA) plates (Statens Serum Institute, Copenhagen, Denmark). A standard culture method (ISO 6579:2002/ Amd.1:2007(E)) with some modifications according to recommendations from Nordic Committee on Food Analyses (NMKL) was used [26] for detection of *Salmonella*. The optimum 8 hours incubation time of samples in BPW already described [16], however in this studied we used 12 hours of incubation. The method was carried out to confirm the presence of *Salmonella* in natural contaminated pork meat samples at the slaughter. Briefly, the pork meat samples were collected at slaughter and enriched

(1:10 w/v) in pre-warmed Buffered Peptone Water (BPW, Merck, Darmstadt, Germany) for 12 ± 2 h at 37 ºC. Three drops (~100µl) of the enriched BPW sample were transferred onto Modified Semisolid Rappaport-Vassiliadis (MSRV) plates and further incubated for another 24h at 41.5 °C. Suspected colonies were streaked onto the selective plating media Xylose Lysine Deoxycholate (XLD) (Oxoid, United Kingdom) and Rambach agar (Merck). The plates were incubated at 37 °C for 24h, and the suspected *Salmonella*-positive colonies were transferred to BA plates and confirmed by API 20E (bioMérieux, Marcy l'Étoile, France) and serotyping [27].

Campylobacter strains were resuscitated and selected on Charcoal Cefoperazone Deoxycholate Agar (CCDA) (Oxoid) and grown on BA at 42 °C in microaerophilic conditions (6% O_2, 6% CO_2, and 88% N_2) before use. Other bacterial strains including *Escherichia coli (E.coli), Streptococcus pneumonia, Enterococcus faecalis, Enterococcus faecium, Proteus hauseri, Citrobacter freundii, Yersinia ruckeri* were grown on BA places and incubated at 37°C, while *Arcobacter cryaerophilus*, *Arcobacter butzleri* and *Arcobacter skirrowii* strains were incubated at 15 °C or room temperature.

2.3. DNA preparation

To test the specificity and sensitivity of the method, chromosomal DNA from different bacterial strains (Table 1) was isolated using DNeasy Blood and Tissue kit (Qiagen, Germany). The concentration of the DNA was determined by Nano drop (Thermo Scientific, USA). Two ng/µl of the chromosomal DNA were used to test the performance of the Direct PCR.

2.4. Primers for the Direct PCR

Specific PCR primers for amplification of different *Salmonella* serotypes were either selected from literature or designed based on multiple alignments of *Salmonella* and non-*Salmonella* sequences of individual genes using Primer-BLAST from NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The specificity of hilA gene (GenBank U25352)

from *Salmonella* spp. was checked by NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with the highly similar sequences (megablast) program selection. It was showed that it is only unique to and ubiquitous in *Salmonella enterica*. The primers targeting *hil*A gene were designed on the basis of an alignment to *Salmonella enterica* (GenBank U25352). The primers were selected and compared with all nucleotide collection (nr/nt) organism database available in NCBI BLAST with 100 % query cover and E value of 0.02 for forward primer and E value of 5×10^{-4} for reverse primer. The sequences are shown in Table 2. The primers were synthesized by DNA Technology (Aarhus, Denmark).2.5. Optimization of sample dilution condition for the Direct PCR

In order to evaluate the effect of sample dilution on the efficiency of Direct PCR, 2 µl of pork meat sample enriched in BPW was spiked with *Salmonella* cells and then diluted in PBS buffer at different ratios of 1:1, 1:3, 1:8, 1:10, and 1:100. Two µl of each dilution were used as the template in the Direct PCR reaction. The optimized sample dilution ratio was applied for the rest of the Direct PCR experiments.

2.6. Direct multiplex PCR assay

The Direct PCR was performed with 2 µl of the diluted pork meat sample (1: 10 dilution in PBS) in 10 µl of the Direct PCR mixture containing 200 nM of *hilA* primer, 300 nM of *fliC* primer, 100 nM of *sdf* primer, and 100 nM of *sefA* primer; 1 x Phusion® Human Specimen PCR Buffer and 0.04 U/µl Phusion Human Specimen DNA Polymerase (Thermo Fisher Scientific, USA). The PCR was conducted in PikoReal™ Real-Time PCR System (Thermo Fisher Scientific, USA). The PCR conditions were 98 ºC for 2 minutes following by 38 cycles of 98 ºC for 15 seconds, 60 ºC for 15 seconds and 72 ºC for 1 minute. PCR amplification was confirmed by 2% agarose gels electrophoresis containing SYBR® Safe DNA Gel Stain (Invitrogen, Life Technologies, USA).

2.7. Sensitivity of the Direct PCR

To test the sensitivity of the Direct PCR, two different approaches were selected:

a) *Salmonella* DNA-spiked samples:

The 10-fold serial dilutions of pure DNA from *S.* Enteritidis and *S.* Typhimurium were spiked in pork meat samples enriched in BPW, giving final concentration ranging from 0.01pg/µl to 10ng/µl.

b) *Salmonella* cell-spiked samples:

The 10-fold serial dilutions of *Salmonella* cells were prepared from *S.* Enteritidis (CCUG 32352) and *S.* Typhimurium (Jeo 3979 Jgt.110) stocks (OD_{600} = 0.8, corresponding to 10^8 cells/ml). The *Salmonella* cells were spiked in pork meat samples enriched in BPW to give final concentrations ranging from 10^0 to 10^7 CFU/ml. A non-spiked sample was included as a negative control.

2.8. Comparison of sensitivity of Direct PCR and conventional PCR used at slaughter

The sensitivity of the Direct PCR was compared to the conventional PCR used at slaughter in which *Tth* DNA polymerase was employed. The main purpose for this experiment was to compare the sensitivity of direct PCR and conventional PCR used at slaughter without pre-sample preparation. Both experiments employed the same primers set developed in this study to eliminate the discrepancy from primers. The conventional PCR used at slaughter PCR mixture contained 0.06 U/µl of *Tth* DNA polymerase (Roche Applied Science, Mannheim, Germany), 1× of PCR buffer (Roche Applied Science, Mannheim, Germany), 200 nM of *hilA* primer, 300 nM of *fliC* primer, 100 nM of *sdf* primer, and 100 nM of *sefA* primer, 500 µM deoxynucleoside triphosphate with dUTP (Applied Biosystems, Foster city, CA), 8% glycerol (Merck, Darmstadt, Germany), 4.0 mM $MgCl_2$ (Roche Applied Science), 2% dimethyl sulfoxide (Sigma, Steinheim, Germany), and 1 g/liter bovine serum albumin (Roche Applied Science). The PCR conditions were 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 seconds, 60 °C for 60 seconds, and 72 °C for 30 seconds. The PCR reaction was performed using a thermocycler (T3 Thermocycle Biometra, Göttingen, Germany). The PCR

amplicon was detected on 2% agarose gels electrophoresis containing SYBR® Safe DNA Gel Stain (Invitrogen, Life Technologies, USA). The conventional PCR used at slaughter was performed in conventional thermo cycler instead of the PikoReal PCR system because PCR reaction with the *Tth* DNA polymerase using the PikoReal PCR system always gave negative results (data not shown). The reason might be the processivity of *Tth* DNA polymerase is not compatible with the fast ramp rate in PikoReal™ PCR System. Therefore, the conventional PCR used at slaughter was performed in conventional thermos cycler model to get the highest efficiency.

2.9. Evaluation of the developed Direct PCR method using naturally contaminated pork meat samples enriched in BPW at slaughter

A total number of 82 pork meat samples (25g for each) were collected at slaughter and enriched in 225 ml Buffered Peptone Water (BPW) (Danish Crown, Herning, Denmark). The samples were transferred to the laboratory in 250 ml Dispatch Container Nunc (Life Technologies, Nærum, Denmark). On arrival, the sample were subjected immediately to laboratory processing or stored at 5°C before testing. All the samples were tested for *Salmonella* by the modified conventional bacterial culture (ISO 6579), TaqMan real-time PCR [26] and the multiplex Direct PCR assays.

2.10. Statistical analysis

A comparative trial between the multiplex Direct PCR and the conventional bacterial culture (ISO 6579) was designed and conducted according to the MICROVAL protocol [28], relative sensitivity, specificity and accuracy were calculated according to the following formulas:

1) Relative accuracy: AC = $\frac{(PA+NA)}{N}$ ×100%

2) Relative specificity: SP = $\frac{NA}{N-}$ ×100%

3) Relative sensitivity: SE = $\frac{PA}{N+}$ ×100%

where:

PA is the positive agreement between the culture and the Direct PCR methods;

NA is the negative agreement between the culture and the Direct PCR methods;

PD is the false positives of the Direct PCR method;

ND is the false negatives of the Direct PCR method;

N is the total number of samples (NA+PA+PD+ND);

N- is the total number of negative results with the reference method (NA+PD) and

N+ is the total number of positive results with the reference method (PA+ND)

Cohen's kappa statistic was performed to assess the agreement between direct PCR, conventional culture method and conventional PCR used at slaughter. The values of 0.00 to 0.20 indicate poor agreement, 0.21 to 0.40 indicate fair agreement, 0.41 to 0.60 indicate moderate agreement, 0.61 to 0.80 indicate good agreement, and 0.81 to 1.00 indicate excellent agreement.

3. Results and discussion

3.1. Development of multiplex Direct PCR for *Salmonella* detection

3.1.1. Selectivity of target gene primers

Previously, a real-time multiplex PCR method has been developed to detect *Salmonella* and differentiate different *Salmonella* serotypes (*S.* Typhimurium, *S.* Enteritidis, *S.* Dublin, *S.* Gallinarum, *S.* Kentucky) in chicken meat [29]. Four primer pairs targeting *fliC* gene, *sdf* gene, *sefA* gene and *aceK* gene have been described (Table 2). In this study, initial experiments were carried out to test the specificity of the four primer pairs for differentiating *Salmonella* and non-*Salmonella* reference strains in pork meat samples. A total of 31 bacterial strains including 15 *Salmonella*

225 serotypes and 16 non-*Salmonella* bacterial strains were tested (Table 1). The experiments revealed
226 that the *fliC* gene primers were able to detect *S.* Typhimurium specifically, the *sdf* gene primers were
227 specific to *S.* Enteritidis and the *sefA* gene primers were specific for *S.* Enteritidis, *S.* Dublin and *S.*
228 Paratyphi A serotypes. The results were in agreement with the results described previously [29]. The
229 *fliC* gene targets i-antigen specific phase 1 flagellin which is also expressed in uncommon serotypes
230 such as Aberdeen, Bergen and Kedougou [29]. It was showed that *fliC* gene is positive in real time
231 PCR for 17 samples of *S.* Typhimurium and negative for 45 non-Typhimurium (both *Salmonella* and
232 non-*Salmonella*) samples [30]. The *sdf* gene was also shown to be able to clearly distinguish *S.*
233 Enteritidis from 73 non-*S.* Enteritidis isolates including 34 different serovars such as Dublin and
234 Pullorum that are very close relative to Enteritidis [31]. In another study, by using PCR targeting
235 *sefA* gene, *S.* Enteritidis and *S.* Senftenberg strains could be identified from a total of 101 strains of
236 bacteria consisting of 14 *Salmonella* spp. and 10 non-*Salmonella* serovars. [32]. This showed that
237 *fliC, sdf* and *sefA* genes can be used for detection of *Salmonella* with different serotypes. The *fliC, sdf*
238 and *sefA* genes are single copy gene and are located on the chromosome.

239 However, when we used the *aceK* gene primers to detect *Salmonella* genus in pork meat samples
240 enriched in BPW, there were a number of false positive PCR (data not shown). The *aceK* gene have
241 previously been shown to be specific for detecting *Salmonella* spp. in chicken samples [29], but this
242 was not the case for the enriched pork meat samples. This discrepancy could be explained by the fact
243 that the microflora and fauna in pork is different from poultry, therefore primers targeting *aceK* gene
244 might falsely identify other species than *Salmonella* spp. in pork meat samples enriched in BPW.
245 Therefore a new primer pair targeting *Salmonella* spp. for the multiplex Direct PCR is needed. The
246 *hilA* gene of *Salmonella* is known to be responsible for the regulation of the Type III secretion
247 system (T3SS) [33], in cell invasion and in causing systemic infection. It is located in pathogenicity
248 island (SPI-1) and real-time PCR targeting *hilA* gene can differentiate 57 different *Salmonella* strains

and 30 non-*Salmonella* strains [17]. The gene has been shown to be a potential candidate for PCR reaction in research, clinical diagnostic and industrial for the detection of *Salmonella enterica* subspecies Enterica [10]. Based on the *hil*A sequence data from Genbank (U25352), we designed a new *hil*A primer pair targeting *Salmonella* genus. The new *hil*A gene primers targets a 225 bp-region starting from the position 1241 bp of the *hil*A gene. The new hilA gene primer pair was used together with the other three primer pairs (*sefA, sdf* and *fliC*) in the developed Direct PCR (Table 2). The multiplex Direct PCR was able to identify and differentiate 15 different *Salmonella* serotypes from 16 non-*Salmonella* bacteria strains (Table 1).

3.1.2. Optimization of sample dilution condition for the Direct PCR.

In this study, the robustness of the *Pfu* DNA polymerase was evaluated using raw pork meat samples enriched in BPW. The enriched meat sample is a complex food matrix which contains high background of normal microbiota and microflora [34] as well as a high concentration of PCR inhibitors. Monteiro et al. [35] reported a simple strategy to reduce the effects of PCR inhibitors by diluting the sample. Hence, the pork meat samples were diluted in PBS buffer at different ratios (1:1, 1:3, 1:8, 1:10, and 1:100) in order to optimize the condition for the Direct PCR (Table 3).

The effect of dilution ratio on the efficiency of Direct PCR is shown in Fig 1. When 2 µl of the raw and 1:1 diluted pork meat samples enriched in BPW were used as template for the Direct PCR, no PCR inhibition effect was observed in most of the cases. However, PCR results were inconsistent when performing the multiplex Direct PCR with samples that contain *Salmonella* serotype 4,5,12:i:- (Jeo 297 Jgt.110) and *S.* Dublin (H 64004) (Table 3). High concentration of potential PCR inhibitors from the sample might be the cause of this inconsistency, since more reproducible results were observed after dilution of 1:3 (Table 3). The highest PCR efficiency was achieved at the dilution

1:10, whereas the amplification efficiency decreased with further dilutions (1:100). Hence the dilution ratio of 1:10 was used for the multiplex Direct PCR in the following experiments.

3.2. Detection limit of the Direct PCR

To determine the sensitivity of the multiplex Direct PCR, two different approaches were used: 1) the use of DNA-spiked pork meat samples enriched in BPW; and 2) the use of *Salmonella* cell-spiked pork meat samples enriched in BPW. Fig 2 and Fig 3 show the results of such experiments in gel electrophoresis. The limit of detection (LOD) of the multiplex Direct PCR using the DNA spiked samples was 100 pg DNA for both *S.* Enteritidis and *S.* Typhimurium (Fig. 2a and 2e). For the bacterial cell spiked samples, the LOD of the Direct PCR method for both *S.* Enteritidis and *S.* Typhimurium was~10^5 CFU/ml (Fig. 3a and 3e).

The Direct PCR was compared to the conventional PCR used at slaughter in terms of sensitivity. In the conventional PCR at slaughter, *Tth* DNA polymerase from thermophilic eubacterium *Thermus thermophilus* HB8 was used. The *Tth* DNA polymerase showed better performance than other DNA polymerase such as *Taq* DNA polymerase [36]. In this study, the sensitivity of both the conventional PCR at slaughter and the Direct PCR were tested using *Salmonella* pure DNA (Fig 2 a, c, e and g) and DNA spiked pork meat sample enriched in BPW (Fig 2 b, d, f, and h). The LOD of the Direct PCR using *Salmonella* pure DNA (without any PCR inhibitor) was determined as low as 1 pg DNA for both *S.* Enteritidis (Fig. 2a) and *S.* Typhimurium (Fig. 2e); while for the conventional PCR at slaughter, the LOD was determined to be 100 pg for both *S.* Enteritidis (Fig. 2c) and *S.* Typhimurium (Fig. 2g). When DNA-spiked pork meat samples were used as templates, the LOD of the Direct PCR was 10 pg for *S.* Enteritidis (Fig. 2b) and 100 pg for *S.* Typhimurium (Fig. 2f), whereas no PCR amplification was observed for either *S.* Enteritidis (Fig. 2d) or *S.* Typhimurium (Fig. 2h) in the conventional PCR at slaughter. In summary, the LOD of the Direct PCR was 100

294 folds lower than that of the conventional PCR at slaughter when using pure DNA. In contrast, when
295 testing the two methods with the DNA spiked BPW samples, the LOD of the Direct PCR was 1,000-
296 10,000 time lower than that of the conventional PCR at slaughter.

297 The sensitivity of the Direct PCR and the conventional PCR at slaughter were also tested using
298 pure *Salmonella* cells (Fig 3 a, c, e, and g) and *Salmonella* cell- spiked enriched pork meat samples
299 (Fig 3 b, d, f, and h). The LOD of the Direct PCR was determined to be as low as 10^2 CFU/ml for
300 both *S.* Enteritidis cells (Fig. 3a) and *S.* Typhimurium (Fig. 3e), while for the conventional PCR at
301 slaughter the LOD was ~ 10^6 CFU/ml for *S.* Enteritidis (Fig. 3c) and ~ 10^5 CFU/ml for *S.*
302 Typhimurium (Fig. 3g), respectively. In contrast, when using spiked meat samples, no PCR
303 amplification was observed for both *Salmonella* serotype (Fig. 3d and h) in the conventional PCR at
304 slaughter, while with the Direct PCR, a LOD of 10^4 CFU/ml were archived (Fig. 3b and f).
305 Therefore, the LOD of the Direct PCR is 1,000 – 10,000 times lower than that of the conventional
306 PCR at slaughter, suggesting that the *Pfu* DNA polymerase has higher amplification efficiency than
307 *Tth* DNA polymerase. Moreover, a shorter total reaction time of 43 minutes was archived for the
308 Direct PCR in comparison to 138 minutes reaction time of the conventional PCR at slaughter. The
309 shorter reaction time of the Direct PCR was attributed to the high processivity of the *Pfu* DNA
310 polymerase that allowed faster amplification as well as removal of the extension step of PCR
311 reaction.

312 This direct PCR protocol performed on PikoReal™ Real-Time PCR System is readily adaptable to
313 a Lab-on-a-chip system which also has fast ramping rate. The advantages of the *Pfu* DNA
314 polymerase, such as the higher tolerant to PCR inhibitors, the ability to omit the sample purification
315 step, faster amplification and the short reaction time, make the Direct PCR a suitable method for
316 online or at site *Salmonella* screening at food production industry. The legislative demand of
317 *Salmonella* detection is 1 CFU/25g sample. In case 1 *Salmonella* cell present in the sample (25g),

318 according to Zheng et al. [37], after 12-hr of enrichment in BPW buffer, the average number of
319 *Salmonella* will reach around 10^6-10^7 CFU/ml. With this range of bacterial concentration, the LOD
320 of ~10^4 CFU/ml of the developed multiplex Direct PCR method is more than sufficient to meet the
321 legislative requirements.

322 3.3. Evaluation of Direct PCR using naturally contaminated pork samples

323 The developed multiplex Direct PCR was used for on-field testing of the pork meat samples at
324 slaughter. A total of 82 pork meat samples enriched in BPW at slaughter were collected and tested
325 using the multiplex Direct PCR. Of these 82 samples, 40 were positive for *Salmonella* spp. and 42
326 were negative for *Salmonella* (Table 4). Among the 40 *Salmonella* positive samples, 24 were positive
327 for both *hil*A and *fli*C and were defined as *S.* Typhimurium; while 14 samples were positive for *hil*A
328 genes and were determined as *Salmonella* spp., and 2 other samples positive for both *hil*A and *sef*A
329 genes were determined as *S.* Dublin. These 82 samples were also tested using standard bacterial
330 culture (ISO6579) combined with *Salmonella* serotyping. The results showed 41 samples were
331 positive and 41 were negative for *Salmonella*. Among the 41 *Salmonella* positive sample, the
332 serotyping revealed that 27 were identified as *S.* Typhimurium, 11 were Derby and 3 were *S.* Dublin.

333 In total, 78 out of the 82 samples identified by the Direct PCR method agreed well with the
334 standard bacterial culture (ISO 6579) on the presence of *Salmonella* as well as the corresponding
335 serotyping. In addition, for the 11 isolates that were identified as *S.* Derby by bacterial culture in
336 combination with serotyping, all were positive for *hil*A gene using the Direct PCR and were thus
337 identified as *Salmonella* spp. For further evaluation, it is important to include target gene that is able
338 to identify *S.* Derby. Four samples showed discrepancy between the two methods. Three samples
339 were identified as *S.* Typhimurium according to the bacterial culture and serotyping, whereas they
340 were identified as *Salmonella* spp. by the multiplex Direct PCR since positive PCRs were only

observed for the *hil*A gene primers (Table 4). Therefore, 14 samples identified as "other *Salmonella* spp." by the Direct PCR actually consisted of 11 *S.* Derby samples and 3 *S.* Typhimurium samples. One sample was identified as *S.* Dublin by bacterial culture, but negative for the *sef*A gene by the multiplex Direct PCR. Using the formulas described in Section 2.10, relative accuracy, sensitivity and specificity of 98.8%; 97.6% and 100%, respectively, were achieved for the multiplex Direct PCR (Table 5). The Cohen's kappa test showed excellent agreement between direct PCR, conventional culture method and TaqMan real-time PCR (Cohen's kappa = 0.81). The cost of single direct PCR is around 0.9 USD (only cost of buffer), therefore it can be another alternative for the industry. Lastly, we suggest including an internal amplification control (IAC) in PCR reaction for future evaluation in the industry. However, the influence of IAC should take into consideration since it may compete with target genes.

4. Conclusion

In this study, by combining two strategies - the use of the *Pfu* DNA polymerase and sample dilution, we developed a new multiplex Direct PCR for rapid and multiplex detection of different *Salmonella* serotypes directly from BPW enriched pork meat samples without DNA isolation and purification steps. The Phusion *Pfu* DNA polymerase showed high resistance to PCR inhibitors in food matrix. The method enabled rapid detection and differentiation of different *Salmonella* serotypes in one reaction within 43 minutes (PCR reaction time only) or 14 hours when including 12h of enrichment time. The new multiplex Direct PCR was used to detect *Salmonella* at sub-species directly from 82 pork meat samples enriched in BPW at slaughter and compared to conventional bacterial culture in combination with serotyping. Relative accuracy of 98.8% with a sensitivity of 97.6% and specificity of 100%, were achieved. The Direct PCR method possesses potential to be used by the food industry and regulatory bodies to monitor food quality and security with much reduced time and cost. Moreover, owing to the rapid and easy manipulation, the developed Direct

PCR is ideally suitable for miniaturization and integration into a Lab-on-a-chip system for online foodborne pathogen detection [38-39].

Conflict of interest

The authors declare that there is no conflict of interest.

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