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Comparative of conventional dot blot hybridization and CARD dot blot hybridization for *Salmonella* detection in pork

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Abstract

Dot blot hybridization assay was evaluated with Sal3 probe for rapid detection of Salmonella from pork samples. The Sal 3 probe (-5′OH) specificity with dot-blot hybridization found a DNA positive result of all salmonella serovars (*S. typhimurium, S. enteritidis, S. vichow*): while, there was negative result from 9 DNA samples of the negative control group. The conventional dot-blot hybridization methods (method A: System probe labeled DIG at 5′-OH labeling DIG hybrids/ anti DIG-AP, detection with NBT/BCIP and method B: System probe labeled with biotin at 5′-OH labeling biotin hybrids / streptavidin-HRP, detection with DAB) were compared with an application of catalyzed reporter deposition (CARD) to dot blot platform (method C: System probe labeled with biotin at 5′-OH labeling biotin hybrids /1°streptavidin-HRP /2°streptavidin-HRP,+ system tyramide signal amplification (TSA), detection with DAB). The sensitivity of dot-blot hybridization methods for systems A, B and C were found C system has a high sensitivity for dot-blot hybridization method. The results were obtained at the lowest concentrations of 3×10^4 cfu / ml using a 2-day examination period. So, the separation processing of pathogens from meat samples is therefore essential. It is recommend that the use of appropriate DNA extraction kits or methods is critical for successful and valid CARD dot blot hybridization posed a challenge for salmonella detection on pork samples.

Keywords: Dot-blot hybridization method, *salmonella spp.*, pork

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1. Introduction

The prevalence of salmonellosis incidence in people of industrialized countries is often implicated severe serotypes such as *S. enteritidis, S. typhimurium* and salmonella 1,4, (5), 12. : i: - [1 – 4]. The salmonella can be adaptive in animal foods and can be transmitted to infectious agents. There is a wide range of living environments both in humans and in pigs [5]. Pork is one of the main animal products that transmit salmonella to the population in EU and USA [1], [2], [6 – 8]. In Thailand, the prevalence of salmonella in swine reported a swine carcass (27.1%), water (36.7%), workers (19.5%) and slaughterhouses (10.7%) respectively [9]. The bacteria culture is gold standard method of bacteria detection in food, which is both a time-consuming conventional culture method and labor-intensive [10]. Molecular methods used to detect organisms are reliable and rapid than traditional methods, involving culture methods or microscopy. Several researchers have developed new screening methods for alternative methods of detecting and enumerating pathogens in food within 1 day including enzyme-linked immunosorbent assay (ELISA), PCR, biosensor and nucleic hybridization technique such as fluorescence *in situ* hybridization (FISH) [11 – 16]. Recently, FISH method was developed to detect the genetic material of salmonella in pig meat. It was found that salmonella could be detected 3x 10^4 cfu/ml of salmonella cells within 1 day, When comparing to accuracy salmonella results between FISH method and bacteria culture (ISO 6579) from pork in the slaughterhouse, both results were accuracy consistent at moderate level (Kappa Statistics = 0.46) [17]. However, these two methods are not feasible in most clinical laboratories in developing countries. Currently, strategies based on dot blot hybridization and an application using the catalyzed reporter deposition (CARD) to dot blot platform (CARD-dot blot hybridization) with rRNA-targeted oligonucleotide probes. CARD

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is a method developed to increase the signal strength of reporter molecules. CARD utilizes the property of peroxidase to catalyze the deposition of tyramide conjugates at the site of enzyme reaction [18]. It may be another alternative method that requires improved approaches to enable specific and sensitivity screening to salmonella and can be conducted using common equipment biotechnology laboratories. Therefore, we developed a dot blot platform and combined with the signal amplification method, CARD to rapidly assess for detection of salmonella in pork. Lastly, we evaluated its efficiency when compared to conventional dot blot hybridization analysis.

2. Material and Method

2.1 Reference strains, culture and DNA extraction

All bacteria isolates were further clinical isolates identified and characterized from genus to species by the Kamphaengsaen Veterinary Diagnostic Center, Faculty of Veterinary Medicine, Kasetsart University, Thailand. *Salmonella enteritidis, Salmonella Typhimurium* and *Salmonella Virchow* were grow as a reference strain and campylobacter spp., *Corynebacterium* spp., *Escherichia coli*., *Klebsiella spp*., *Pseudomonas* spp., staphylococcus aureus., Aeromonas hydrophila and Enterobacter aerogenes were grown as a negative control. The reference strain of *Salmonella* spp., and negative bacteria were streaked into MAC agar plates for over-night culture at 37°C for activating bacteria. 1 – 2 colony of each bacterial were picked and inoculated each into a 5 ml nutrient broth and grew overnight at 37°C with Orbital Shaker (BIOSAN, LATVIA) before harvesting cell at logarithmic phase to obtain cells with high ribosome content for DNA extract. Genomic DNA extraction of all isolates was performed using commercially available the E.Z.N.A. Bacterial DNA Kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's instructions and the concentrations of genomic DNA samples were measured using a Nano-Drop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States) and the concentration was adjusted to 100 μg/ml for test specificity of Sal3 probe determined by dot-blot hybridization.

2.2 Oligonucleotide probe

A specific oligonucleotide probe for dot-blot hybridization was used Sal3 primer (5′-AATCACTTC ACCTACGTG-3'), specific to target the 23S rRNA for *Salmonella spp*. [16], [19 – 23]. The Sal3 probe were all synthesized and labelled with digoxigen in (Sal3-digoxigenin) and the biotin (Sal3-Biotin) at the 5′end (Asia Pacific. Integrated DNA Technologies, Singapore).

2.3 Evaluation of the specificity of Sal3 probe by dot blot analysis

The specificity of the Sal 3 probe was tested by hybridization with dot-blot hybridization assay. For this study, Sal3 probe was tested against *Salmonella* DNA 3 serovars and 9 negative controls.

The pellet DNA of each bacterium was diluted to a concentration of 100 μg/ml, DNA boiling for 5 minutes at 100°C and placing on ice for 10 minutes. Four μl volumes of DNA sample were dotted on nitrocellulose membrane and dried at 80°C for 2 hr in hot air oven. Dot-blot hybridization in this study followed with the procedure of [24]. Nitrocellulose membrane were sealed in a polypropylene bag with, per cm^2, 1 ml of a prehybridization mixture containing 20xSSC (3M NaCl, 0.3M Na-Critrate), 50x Denhardt,s solution, yeast tRNA, 1M $Na_2HPO_47H_2O$, 10% Dextran sulfate) that were incubated at 37°C for 2 hr in water bath. After that prehybridization mixture was removed and replaced with mixed probe/hybridization buffer solution (4 μl: 500 μl). The membranes were incubated in water bath at 37°C overnight and membrane was carefully washed away with consecutive washes in wash buffer 1 (1xSSC, 0.1%SDS, pH 7) at 37°C for 5 minutes, wash buffer 2 (0.1xSSC, 0.2%SDS, pH 7) at 37°C for 5 minutes and in wash buffer 3 (0.5x SSC, pH 7) at 37°C for 1 hr. After that blocking reagent with skim milk (5% in 1xTris-buffered saline, TBS) was added and applied to the membrane for 1 hr at room temperature and wash membrane with 1xTBS, pH 7 for 5 minutes at room temperature. In detection step, the membranes were incubated at room temperature for 1 hr with anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (diluted 1:100 in 3% BSA, Roche Diagnostics, German) and washed three times with 1xTBS, each time for 5 minutes. Alkaline phosphatase substrate NBT/BCIP (nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolyphosphate, Thermoscientific) was added to membrane. The development of a dark blue positive reaction was allowed to proceed for 20 minutes at room temperature and membrane was washed for 5 minutes with 1xTBS buffer, air dried and stored in a polypropylene bag.

2.4 Analytical sensitivity of the methods

The sensitivity of conventional dot-blot hybridization methods (method A: System probe labeled DIG at 5′-OH labeling DIG hybrids/ anti DIG-AP, detection with NBT/BCIP and method B: System probe labeled with biotin at 5′-OH labeling biotin hybrids / streptavidin-HRP, detection with DAB), were compared with an application of catalyzed reporter deposition (CARD) to dot blot platform (method C: System probe labeled with biotin at 5′-OH labeling biotin hybrids/ 1°streptavidin-HRP/ 2°streptavidin-HRP, + system tyramide signal amplification (TSA), detection with DAB).

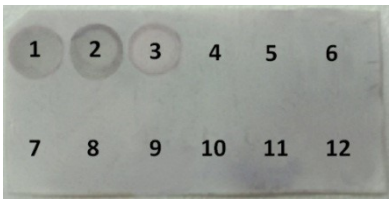


Figure 1: Determination of specificity of Sal3 probe by dot blot hybridization. DNA of Salmonella (1 = *S. enteritidis*, 2 = *S. typhimurium* and 3 = *S. paratyphimurim*). DNA of negative bacteria (4 = Actinomyces spp., 5 = Campylobacter jejuni., 6 = Corynebacterium spp., 7 = Escherichia coli., 8 = Klebsiella spp., 9 = Pseudomonas spp., 10 = Staphylococcus aureus., 11 = Streptococcus agalactiae and 12 = Streptococcus suis)

Method A: DNA extracted from *S. enteritidis* of each tenfold dilution sequence was tested for the sensitivity of dot blot hybridization, using a Sal 3 probe (labeled DIG at 5′-OH) and detection step according to step 2.3

Method B: DNA extracted from *S. enteritidis* of each tenfold dilution sequence was tested for the sensitivity of dot blot hybridization, by following per under step 2.3 using a Sal 3 probe (biotin at 5′-OH labeling) and detection step. 3 – 4 drops of the diluted Primary Streptavidin-HRP solution (GenPoint^{TM}, DAKO) were applied to cover the membrane and incubate at room temperature for 30 minutes. Membranes were rinsed in TBST wash buffer and placed in three fresh TBST wash buffer baths for 5 minutes each to remove residual primary streptavidin-HRP solution. 3 – 4 drops of diluted DAB chromogen were applied to cover the membrane and incubated at room temperature for 5 minutes, which contrasted well with the brown DAB signals and stopped the chromogen reaction by immersing membrane in water for 1 minute, air dried and storing in a polypropylene bag.

Method C: DNA extracted from *S. enteritidis* of each tenfold dilution sequence was tested for the sensitivity of dot blot hybridization, by following per under step 2.3 using a Sal 3 probe (biotin at 5′-OH labeling) and detection was performed using the Dako GenPoint^{TM}, 3 – 4 drops of the diluted Primary Streptavidin-HRP solution were applied to cover the membrane and incubated at room temperature for 15 minutes. Membranes were rinsed in TBST wash buffer and placed in three fresh TBST wash buffer baths for 5 minutes each to remove the residual primary streptavidin-HRP solution. Catalyzed signal amplification method (CARD) for biotinylated probes. Briefly, 3 – 4 drops of biotinyl tyramide were applied to cover the membrane and incubated at room temperature for 15 minutes. Membranes were rinsed in TBST wash buffer and placed in three fresh TBST wash buffer baths for 5 minutes each to remove residual biotinyl tyramide solution. 3 – 4 drops of secondary streptavidin-HRP solution were applied to cover the specimen and incubated at room temperature for 15 minutes. Membranes were rinsed in TBST wash buffer and placed in three fresh TBST wash buffer baths for 5 minutes each to remove residual secondary Streptavidin-HRP solution. After that, 3 – 4 drops of diluted DAB chromogen were applied to cover the membrane and incubated at room temperature for 5 minutes, which contrasted well with the brown DAB signals and stopped the chromogen reaction by immersing membrane in water for 1 minute, air drying and storing in a polypropylene bag.

2.4.1 Evaluation of detection limit of dot blot hybridization of three systems (A, B and C) in pure culture

S. enteritidis was the culture in 5 ml of trypic soy broth (TSB), incubate at 37°C for 18 – 24 hr., at a concentration of McFarland turbidity standard of 1 (Grantbio, U.K.), which was approximately 3×10^8 cfu/ml. Suspended cultures were diluted serially in 10-fold steps. Each serials dilution of bacteria sample were performed as DNA was extracted described previously and measurement of DNA concentration (Nanodrop, Thermoscientific) as described above to determine the sensitivity of all three different detection methods of dot blot hybridization, according to 2.3. One of the systems was chosen to detect the lowest amount of salmonella to continue for the next steps (2.4.2).

2.4.2 Sensitivity of dot-blot hybridization in spiked pork

Hygienic pork obtained from the supermarket was confirmed as without contamination of external microorganisms (Charoen Pokphand Foods PCL (CP Foods), Thailand). Pork meat spiked with different concentrations of *S. enteritidis* that made tenfold dilution according to step 2.4.1. One milliliter of each serial dilutions of *S. enteritidis* was spiked on 25 g of pork meat that was added to 25 ml BPW with 0.1% Tween 80 solution and homogenized with a stomacher (BagMixer®400W) at high speed for 90 s. The 25 ml of bacteria cells were collected by filtration through 33-μm pore-size nylon screen mesh (diameter, 25 mm) into centrifuge tubes 50 ml, and then centrifuged at 7,000 rpm for 20 minutes. The BPW solution of each serial dilution was poured and dissolved the microbial sludge with 2 ml PBS and vortex at high speed for 5 minutes. So, one milliliter of each samples was centrifuged at 8,000 rpm for 10 minutes and cell pellet was extracted DNA as previously described for exam-

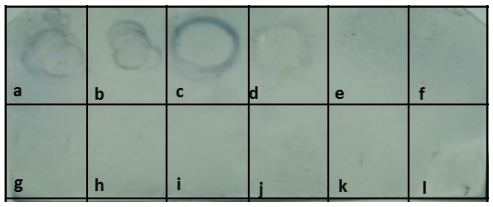
ining the sensitivity of the CARD-dot blot hybridization [17].

3. Results and Discussion

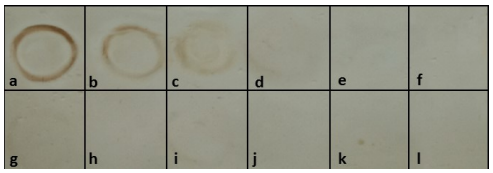
3.1 Evaluation of specificity of Sal3 probe by dot blot analysis

The results of the specificity of the Sal 3 probe was tested by dot blot hybridization. The Sal 3 probe was found to be 100% specific for *S. enteritidis, S. typhimurium and S. Virchow.* (Fig.1), and can be directly determined by observing the Sal3 hybridized probes. The existence of purple dots at salmonella DNA dots at the locations was spotted. But, no cross-hybridization was observed with 9 strains of negative control. It showed that Sal 3 primer labeled with digoxigenin at the end of 5′OH was specific to all 3 *salmonella typhimurium, salmonella Virchow, salmonella enteritidis*, which are serovars important for causing gastrointestinal poisoning in Thai people [25], [26].

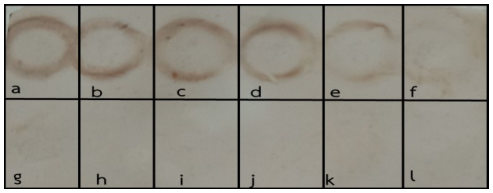
In this study, we have developed a dot blot hybridization assay for the detection of *Salmonella* using Sal3 oligonucleotide probe (5,-ATCACTTCACC TACGTG-3,). The DNA target was hybridized with the membrane immobilized probe and the hybridization was detected by chemiluminescence. 23S rRNA gene of *Salmonella* from 3 different serovars of *Salmonella* hybridized with the probes was found, whereas those of species of *Actinomyces* spp, *Campylobacter jejuni*, *Corynebacterium* spp, *Escherichia coli*, *Klebsiella* spp, *Pseudomonas* spp, Staphylococcus aureus, Streptococcus agalactiae, and Streptococcus suis failed to hybridize. The specific probe binding in this study indicated hybridized Sal3 probe of 100% all salmonella strains. No cross-reaction to other strains of the Enterobacteriaceae family was observed (Fig.1). Nordentoft [19] has been published, that Sal3 oligonucleotide probes designed from the base sequence of the 23S rRNA gene (rDNA), salmonella-specific. The rRNA region has databases from domain or other higher taxa down to a species and rRNA was a routine region used for species identification. Moreover, species-specific probes designed from these genes can be applied for the analysis of any community. They can be detected using whole cell methods in which the cell remains intact and thus also the morphology, or using cell free methods in which total nucleic acids were extracted and probes were applied directly to the nucleic acid target [27 – 29]. A previous study showed that a Sal3 probe has been used for the detection of salmonella in food, wastewater [19], [20], [30 – 32]. The sequence of Sal3 probes was complementary with the helix 63 regions of 23S rRNA gene of salmonella [19]. The region was a highly conserved region of salmonella DNA among different species [33], [34].



1. A-DIG: System probe labeled DIG at 5′-OH labeling / anti DIG-AP, detection with NBT/BCIP



2. B-BIOTIN: System probe labeled with biotin at 5′-OH labeling/ streptavidin-HRP, detection with DAB.



3. C-CARD: System probe labeled with biotin at 5′-OH labeling / 1°streptavidin-HRP/ 2°streptavidin-HRP, +system tyramide signal amplification (TSA), detection with DAB

Figure 2: Comparative dot blot analysis determining the sensitivity of three methods for the visualization of dilution factors of salmonella DNA with enhanced haptens signal as chromogen: Dilution factor of *Salmonella* cell was DNA extraction: a= Initial concentration~10^8cfu/ml, b = -1, c = -2, d = -3, e = -4, f = -5, g= -6, h = -7, i = -8, j = *Escherichia coli*, k = *Aeromonas hydrophila*, and l = *Enterobacter aerogenes* (negative control)

3.2 Evaluation of detection limit of dot blot hybridization of three methods (A, B and C) in pure culture

To determine the lower limit of dot blot hybridization assay, ten-fold serially diluted (10^0 – 10^8); then, we tested the 3 methods of detection by dot blot hybridization assay established in this study for simultaneous detection of *Salmonella* spp. As a consequence of conventional dot blot hybridization assay such as method A: System probe was labeled DIG at 5′-OH labeling DIG hybrids/ anti DIG-AP, detection with NBT/BCIP and method B: System probe was labeled with biotin at 5′–OH labeling biotin hybrids /streptavidin-HRP, detection with DAB), the detection limit in pure culture of *S. enteritidis* was determined to be 10^6 cfu/ml (DNA concentration 22.40 μg/μl; expected cell concentration = $<\sim 3\times10^3 - 10^4$ cfu/ml). However, method C: System probe was labeled with biotin at 5′-OH labeling biotin hybrids /1°streptavidin-HRP /2°streptavidin-HRP, + system tyramide signal amplification (TSA), detection with DAB), the detection limit in pure culture of *S. enteritidis* was determined to be 10^4 cfu/ml (DNA concentration 9.41 μg/μl; expected cell concentration = < 3cfu/ml), as shown in (Fig. 2; Table 1).

Dot blot hybridization assay is a diagnosis tool of routine use in the diagnosis laboratory. When

Table 1. The relationship between serial dilutions and the amount of DNA of *S. enteritidis* extracted from pure culture on dot blot hybridization assays.

Dilution factor	Number of salmonella cell(cfu/ml)	Elute of 30 μl of DNA extraction, concentration (μg/μl)	Wavelength (A260/280)	4μl of DNA concentration were doton membrane (μg/μl)	Expectedcell concentration (cfu/ml)	Observation of Dot blot hybridization assay		
						Method A	Method B	Method C
Initial	3×10^8	220.9	1.85	36.81	$\sim 10^4$	+	+	+
−1	3×10^7	152.45	1.89	25.4	$\sim 3 \times 10^3 - 10^4$	+	+	+
−2	3×10^6	134.45	1.82	22.4	$\sim 3 \times 10^3 - 10^4$	+	+	+
−3	3×10^5	82.4	1.84	13.73	< 3	-	-	+
−4	3×10^4	56.5	1.88	9.41	< 3	-	-	+
−5	3×10^3	21.7	1.71	3.61	< 3	-	-	-
−6	3×10^2	19.2	1.72	3.2	< 3	-	-	-
−7	3×10^1	13.5	1.51	2.25	< 3	-	-	-
−8	3×10^0	14.7	1.6	2.45	< 3	-	-	-

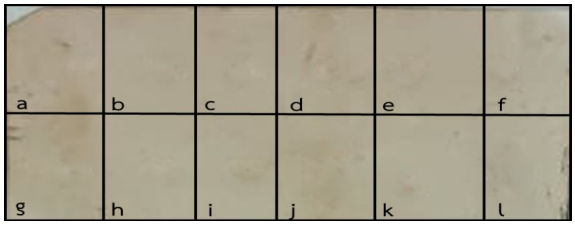


Figure 3: The sensitivity of the salmonella DNA detection limits of CARD dot blot hybridization. : Dilution factor of Salmonella cell: a = Initial concentration$\sim 10^8$ cfu/ml, b = -1, c = -2, d = -3, e = -4, f = -5, g = -6, h = -7, i = -8, j = Escherichia coli, k = *Aeromonas hydrophila* and l = *Enterobacter aerogenes* (negative control)

a pure culture was used, the direct detection methods of digoxigenin probe by anitigoxigenin-alkaline phosphatase conjugate (method A) and biotinylated probe by streptavidin- Horseradish peroxidase conjugate (method B) were compared with the CARD detection method (Method C). The third one proved to more sensitive than the conventional detection using homologous 23S rRNA targets of salmonella. The dot blot hybridization assay using the CARD detection method was able to minimum amount of detectable 9.41 μg/ μl of salmonella DNA corresponding approximately to 10^4 genome copies. CARD detection method based on signal amplification by the tyramide reaction is generally considered the most sensitive of the dot blot hybridization methods; on average their sensitivity is 100 times greater than that of conventional dot blot hybridization. Corresponding, previous studies showed that CARD-FISH methods are more sensitive than the conventional FISH methods [35 – 37]. The sensitivity of the different of assays is in the range of 10^4 – 10^8 genome copies; therefore, even if slightly higher sensitivity can be reached with different method detection (A, B and C), dot blot hybridization assays can detect the total number of salmonella cells. Consequently, dot blot hybridization methods add a signal amplification step (method C), Using tyramide substrates in sequential horseradish peroxidase reactions that described here could be completed within 2 days and was specific for the detection of salmonellae. However, the sensitivity of the CARD dot blot hybridization that was determined uses different serial diluted salmonella cell concentrations of DNA extracted from pork samples. The CARD dot blot hybridization was not detection of all DNA range of spike concentrations of salmonella cells. This suggested that the recovery of salmonella rRNA from pork was not as good as from pure cultures.

By this assay, the method C: was possible to detect in the order of 10^4 salmonella cell of pure culture in 16 – 18 hr. Therefore, we chose method C to test artificial contamination with *S. enteritidis* on pork meat.

3.3 Sensitivity of dot-blot hybridization for salmonella detection on pork spiked

Regarding the sensitivity test for determination of the DNA content from serially diluted salmonella cells in step 2.1 that spiked on pork samples, CARD - Dot blot hybridization by Sal3 probe was not possible to hybridize both with salmonella DNA and negative control of bacterial as shown in fig.3, (Table 2).

Several other methods have been described as specificity and sensitivity of technique detection such as PCR and ELISA for salmonella detection that strongly affected by inhibitory substances which might be present in for example food [38]. For example, fat, glycogen, organic and phenolic compounds and humic acids can cause false-negative results of method detection [39]. The DNA concentration and Purity are important factors for the success of DNA base methods including dot blot hybridization. In generally, the DNA extraction kits are often designed for extracting DNA form pure culture consisting of highly concentrated cells and methods detection that are high sensitivity [40]. When we tested the meat samples diluted

Table 2. Serial dilutions of the relationship and the amount of DNA of *S. enteritidis* extracted from pork spike on CARD dot blot hybridization assay.

Dilution factor	Number of salmonella cell (cfu/ml)	Elute of 30 μl of DNA extraction, concentration (μg/μl)	Wave length (A260/280)	4μl of DNA concentration were doton membrane (μg/μl)	Expected cell concentration (cfu/ml)	Observation of CARD dot blot hybridization
Initial	3×10^8	21.7	1.98	2.89	< 3	-
-1	3×10^7	18.8	1.82	2.51	< 3	-
-2	3×10^6	19.2	1.8	2.56	< 3	-
-3	3×10^5	29	1.79	3.87	< 3	-
-4	3×10^4	9.5	1.82	1.27	< 3	-
-5	3×10^3	14.7	1.82	1.96	< 3	-
-6	3×10^2	13.5	1.86	1.80	< 3	-
-7	3×10^1	4.5	1.57	0.60	< 3	-
-8	3×10^0	5.9	2.81	0.79	< 3	-

with salmonella cell concentrations; it found that low amount of DNA extracted and low purity of DNA extracted (Table 2), which is may be caused by fat tissue from the meat samples (Minced pork with pork fat). The complexities of various food materials and the use of various extraction methods result in different DNA yields [41]. Corresponding to Laube *et al.*, [42] it reported a discrepancy in the yield of DNA extracted from various source of tissues. Fatty tissues produced a lower concentration of DNA compared to kidney, liver, heart and tendon tissues. That may be due, to high debris amounts of fatty tissue in the pork and the difficulties of removing fatty substances during the DNA extraction step. Thus, it is necessary to add steps for pretreated sample appropriate such as fat removal before DNA extraction [43]. In addition, the low DNA recovery in this study may be a loss of DNA during sample processing. This is in accordance with Zhang *et al.*, [44] which reported during samples processing of pathogen detections base on a molecular technique (eg. Filtration, centrifugation, DNA extract). So, this causes underestimated quantification of DNA to be significantly lower. However, detection of CARD dot blot hybridization can detect at a low abundance of salmonella cells in pure culture. But, it's not suitable for pork samples. In future study, we recommend that use of appropriate DNA extraction kits or methods is critical for successful and valid CARD dot blot hybridization which posed a challenge for salmonella detection on pork samples.

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