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RESEARCH ARTICLE

Comparison of bacterial culture plus PCRs and SYBR Green real-time PCR methods in detecting *Arcobacter butzleri* in pork meat with different pre-enrichment periods

Parada Utto[1], Kochakorn Direksin[2*]

[1] *College of Innovation and Management, Songkhla Rajabhat University, Songkhla 90000, Thailand*
[2] *Division of Livestock Medicine, Faculty of Veterinary Medicine, Khon Kaen University, Khon Kean, 40002, Thailand*
[*] ***Corresponding author*** *E-mail: kochakrn@kku.ac.th*

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Abstract

Objectives: A speedy diagnosis of food poisoning bacteria is crucial. Although PCR can detect *Arcobacter* faster than bacterial culture-based diagnosis, sample pre-enrichment (PE) for 48 hours is usually needed to increase the test's susceptibility. This study aimed to find *Arcobacter butzleri* in pork samples and figure out the shorter PE times of pork samples for real-time PCR versus the traditional diagnostic methods.

Materials and Methods: Sixty fresh pork samples were collected from retail meat shops in Mueang Khon Kaen district, Khon Kaen province, from October 2021 to March 2022. This study compared *A. butzleri* diagnosis between bacterial culture plus classical PCRs and SYBR Green real-time PCR. Both methods used the same samples of pork meat without (0 hour) or with pre-enriched samples (PE) for 24 and 48 hours to target species-specific 16S rRNA genes in the bacterium.

Results: Using 10-fold serial concentrations of the reference strain (*A. butzleri* DMST19680), the detection limit of SYBR Green real-time PCR was 2.8 x 10 CFU/mL in bacterial suspension and 2.8 x 10^4 CFU/mL in pork matrix. In sixty pork samples, real-time PCR did not directly detect *A. butzleri* (0 h PE) but did detect it at 90.0% in the 24 h and 48 h PE samples. *A. butzleri* was found at 3.3%, 63.3%, and 85.0% in the 0 h, 24 h, and 48 h PE samples, respectively, by bacterial culture-based methods. Considering 48 h PE as the gold reference, the prevalence of *A. butzleri* in the pork samples was 85.0% and 90.0% when using culture-based and real-time PCR approaches, respectively. Real-time PCR (24 h PE) had 100% sensitivity, 66.7% specificity (95%CI: 35.9–97.5), and 95.0% accuracy (95%CI: 89.5–100). The bacterial culture-based (48 h PE) and real-time PCR (24 h PE) methods had good agreement (kappa = 77.3%; 95%CI: 59.7–94.9).

Conclusions: *A. butzleri* was highly prevalent in pork samples sold in the Mueang district of Khon Kaen. The results proved that SYBR Green real-time PCR with 24 h PE was a reliable screening method for faster detection of *A. butzleri* in pork samples.

Keywords: *Arcobacter butzleri,* bacterial culture, pork, pre-enrichment, SYBR Green real-time PCR

Introduction

The *Arcobacter* genus is an emerging foodborne pathogen in humans, and public health awareness is increasing worldwide (Ramees *et al*., 2017). Some species of this genus are associated with gastroenteritis, bacteriemia, and septicemia in humans (Jiménez-Guerra *et al*., 2020; Soma Sekhar *et al*., 2018; Webb *et al*., 2016). *Arcobacter*, especially *Arcobacter butzleri* (*A. butzleri*), is considered an emerging food poisoning etiology (Jiménez-Guerra *et al*., 2020; Sekhar *et al*., 2017; Webb *et al*., 2016). Reports of the prevalence and novel species of *Arcobacter* are increasing across the globe, especially in countries known for good hygienic practices. There were reports of *Arcobacter* in various sources, including animals, humans, meat products, seafood, vegetables, and water samples (Aydin et al., 2020; González et al., 2017; Kim et al., 2019; Mizutani et al., 2019; Morejón et al., 2017; Niedermeyer et al., 2020; Ramees et al., 2017; Shrestha et al., 2019; Soma Sekhar et al., 2018; Zhang et al., 2019). The common species causing diseases in humans were *A. butzleri*, *A. cryaerophilus*, *A. skirrowii*, and *A. lanthieri* (Brückner *et al*., 2020). *A. butzleri* usually causes the most severe diseases in humans (Jiménez-Guerra *et al*., 2020), and it is found at the highest rate in the food chain (Ferreira *et al*., 2019), especially in swine carcasses (Gobbi *et al*., 2018). Nonetheless, there is limited information about this bacterium in pork meat in Thailand. The few related studies have focused on the presence of *Arcobacter* in tourists, retail food, and water samples in Thailand (Bodhidatta et al., 2013; Morita et al., 2004; Teague et al., 2010; Tomioka et al., 2021; Vindigni et al., 2007) These reports are rather outdated. It is, therefore, important to routinely monitor *Arcobacter* in food, particularly in pork meat.

A prompt diagnosis is critical for tracking infectious food sources. So far, there is no established method for diagnosing *Arcobacter*, and bacterial isolation remains the gold standard (Ramees *et al*., 2017). A selective pre-enrichment (PE) step is necessary for sensitive detection of *Arcobacter* because of concurrent microbiota and the presence of non-specific inhibitors in food samples. SYBR Green real-time PCR has been found to be highly specific and sensitive in identifying *Arcobacter* in chicken products and meat samples (González et al., 2010; Ramees et al., 2014). However, these earlier studies still need selective PE for 48 h before the analysis. To find *Arcobacter* in pork samples, it is necessary to figure out whether a shorter PE time or direct detection is possible. This study finds shorter PE periods for real-time PCR diagnosis versus the traditional method and reveals a high prevalence of *A. butzleri* in pork samples.

Materials and Methods

Sample size estimation and sample collection

A sample size was determined and adopted from previously published data (Bujang and Adnan, 2016). This ready-to-use tabulated data derived from the formulation of sensitivity and specificity tests using Power Analysis and Sample Size (PASS) software (PASS 11. NCSS, LLC, Kaysville, Utah, USA). When the prevalence of *Arcobacter* spp. in pork meat is 50.0% (specifically 55.6%; Kim *et al*., 2019), a minimum number of 40 samples is required to achieve a minimum power of 80.4% for detecting a change in the percentage value of sensitivity of a screening test from 0.50 to 0.80 based on a target significance level of 0.05 (specifically $p = 0.041$).

Fresh pork samples from retail meat shops in municipal areas in Mueang district, Khon Kaen province, Thailand, were collected between October 2021 and March 2022. Every month, 10 samples were collected at random from supermarkets and fresh markets. A total of 60 pork samples were included in the study. Other *Arcobacter*-negative pork was used as a pork matrix for quantitative bacterial inoculation, as confirmed by the bacterial culture and molecular methods.

Reference bacteria and isolation media

A. butzleri DMST19680 from the Department of Medical Sciences, Ministry of Public Health, Thailand, was used as the reference strain for positive control and detection limit evaluation throughout the study. The genomic DNA template of *A. butzleri* DMST19680 was used as a positive control in the assays. *Arcobacter* enrichment broth (AEB) was prepared using the *Arcobacter* basal medium CM0965B (Oxoid, Hampshire, UK) and CAT supplement SR174E (Oxoid). The CAT supplement was composed of cefoperazone (8 mg/L), amphotericin (10 mg/L), and teicoplanin (4.0 mg/L). Modified charcoal cefoperazone deoxycholate agar (mCCDA) was formulated from campylobacter blood-free selective agar base CM0739 (Oxoid) and CCDA selective supplement SR0155E (Oxoid). CCDA has

cefoperazone (32 mg/L) and amphotericin B (10 mg/L). Blood agar (BA) was prepared by adding 5% (v/v) defibrinated bovine blood to BA base CM271 (Oxoid).

Pre-enrichment (PE) periods of pork samples

Ten grams of fresh pork samples were aseptically minced with scissors and suspended in 90 mL of AEB plus CAT supplement (AEB-CAT). The mixtures were homogenized with a stomacher for 1 min. Each pork sample was made in triplicate in AEB–CAT broth for 0, 24, and 48 h PE periods, and the inoculum broth was incubated at 30 °C under aerophilic conditions. The process of sample preparation is illustrated in Figure 1. These different pre-incubation time inoculums were used in *Arcobacter* diagnosis by bacterial culture based or SYBR Green real-time PCR methods.

Arcobacter identification by bacterial culture plus PCRs

For bacterial isolation, 100 µL of a specific PE inoculum (0, 24, or 48 h PE) was transferred onto a 0.45 µm membrane filter (cellulose acetate, 25 mm diameter, Whatman, UK) placed on mCCDA agar plates and allowed to filter passively under ambient conditions for 60 min. Afterward, the filters were discarded, and the agar plates were incubated at 30 °C for 48 h under aerobic conditions. If there was no growth after 72 h, the culture was regarded as negative. Presumptive *Arcobacter* colonies (small, colorless, translucent, and convex with an entire edge) were sub-cultured on BA plates. Pure colonies were subjected to Gram staining, urea, oxidase, and motility testing under a phase-contrast microscope. If the bacterial colonies were gram-negative, spiral, motile, urea-negative, and oxidase-positive (Fernandez *et al.*, 2015), the *Arcobacter* genus and species were confirmed using a classical PCR assays. Steps of *Arcobacter* isolation and identification in pork samples are summarized in Figure 2. The time needed for *Arcobacter* diagnosis in pork samples by bacterial culture plus PCR assays is 5-8 days, and by the SYBR Green real-time PCR method, it is 2-3 days (Figure 2).

Genomic DNA of potential *Arcobacter* colonies was extracted using the QIA Symphony DSP DNA MiDi kit (Qiagen, Hilden, Germany). To identify *Arcobacter* in the culture isolates, classical PCR was conducted using ARCO-Fw and ARCO-Rw primers specific to the *Arcobacter* genus (Table 1), as previously described (González et al., 2014). The details of the genus-specific PCR method were performed as follows. The PCR reaction mixture contained 5 µL of 10 x *Taq* buffer (Thermo Fisher Scientific, USA), 1.5 mM of 25 mM $MgCl_2$ (Thermo Fisher Scientific), 0.2 mM of

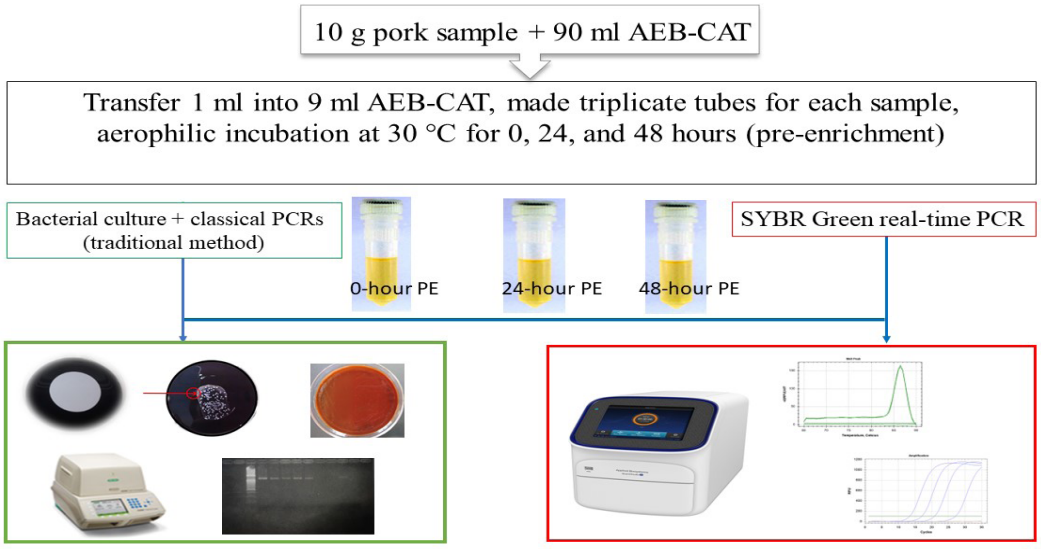

Figure 1. Steps of pork sample processing to assess three different pre-enrichment (PE) periods for bacterial culture plus PCRs and SYBR Green real-time PCR assays.

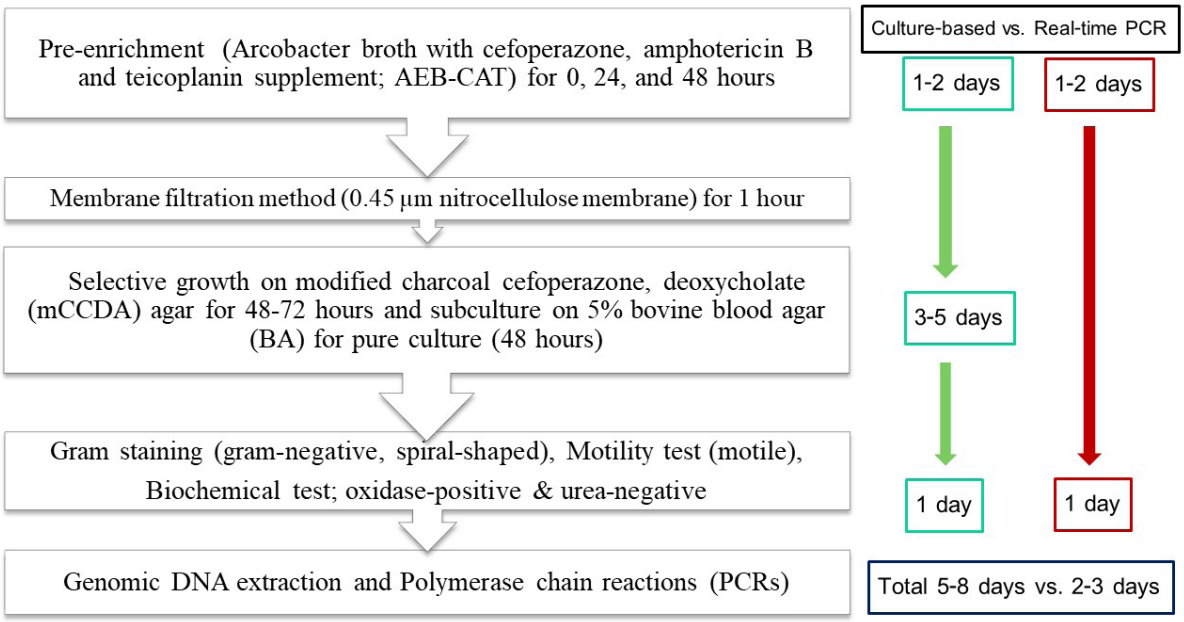

Figure 2. Time required for *Arcobacter* diagnosis in pork samples by bacterial culture plus PCR assays and the SYBR Green real-time PCR method.

10mM dNTPs (Thermo Fisher Scientific), 0.1 µL of each 10 µM primer (Integrated DNA Technologies, Singapore), 1.25 units of 5U Taq DNA Polymerase (Thermo Fisher Scientific, USA), and 5 µL bacterial DNA template. Total volume was adjusted to 50 µL using DNase-free distilled water (Invitrogen, UK). All steps were performed on ice. DNA of the reference strain *A. butzleri* DMST19680 was used as a positive control, and sterile deionized water was used as a negative control. Amplification was run on a MyCycler PCR machine (Bio-Rad, Hercules, CA, USA). After pre-denaturation at 95°C for 5 min, the thermal cycling program was conducted. The program involved 35 cycles of denaturation at 95°C for 30 sec, primer annealing at 56°C for 30 sec, an extension at 72°C for 30 sec, and a final extension at 72°C for 7 min. The amplified products (85 bp) were electrophoresed (3% agarose gel), stained with ethidium bromide, and then visualized under a UV transilluminator (Vilber Lourmat, France).

To indicate *A. butzleri*, a classical species-specific PCR was done using previously designed primers (Houf *et al*., 2000). The PCR Master Mixture composition was the same as in the genus-specific PCR, except for the primers BUTZ-F and ARCO-R (Table 1). After pre-denaturation for 5 min at 95 °C, the following amplification conditions were adopted: 35 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 45 sec, an extension step at 72 °C for 1 min, and a final extension step at 72 °C for 7 min. The PCR products were electrophoresed in a 1.5% agarose gel (Vivantis, Malaysia), stained with ethidium bromide, and visualized under ultraviolet light.

Arcobacter butzleri identification by SYBR Green real-time PCR

At the end of each specific PE period, 600 µL of AEB–CAT inoculum was taken for DNA extraction using the QIA Symphony DSP DNA MiDi kit (Qiagen). The DNA templates and primers (BUTZ-F and ARCO-R) specific for *A. butzleri* (Houf *et al*., 2000) were used in the SYBR Green real-time PCR assay (Table 1). The SYBR Green is a free-floating fluorescent dye that binds to double strand-DNA for quantitative real-time PCR (qPCR). The SYBR Green PCR reaction mixture had 10 µL of the SYBR Green Master Mix (Bio-Rad), 1 µL of each primer (0.2 µM), and 2 µL of DNA template, and the total mixture volume was adjusted to 20 µL using DNase-free distilled water (Invitrogen). A real-time PCR assay was performed in a QuantStudio™ 5

Table 1. Primers specific to *Arcobacter* genus and *A. butzleri* in this study.

Primer name	Target gene	Nucleotide (5′ to 3′)	Product size (bp)	References
ARCO-Fw	16S rRNA	GAG GAT GAC ACA TTT CGG TGC	85	(González et al., 2014)
ARCO-Rv		GGA GTT AGC CGG TGC TTA TTC ATA TA		
BUTZ-F	16S rDNA	CCT GGA CTT GAC ATA GTA AGA ATG A	401	(Houf et al., 2000)
ARCO-R		CGT ATT CAC CGT AGC ATA GC		

Real-Time PCR system (Applied Biosystems, USA) machine with an initial cycle of 94 °C for 4 min, followed by 35 four-step cycles of 94 °C for 60 sec, 56 °C for 45 sec, 72 °C for 1.5 min, and 72 °C for 7 min. The specificity of SYBR Green real-time PCR assay was verified using melting curve analysis at the end of the 35-reaction cycles. The genomic DNA templates from the reference strain, *A. butzleri* DMST19680 solution stocks, were used in SYBR Green real-time PCR to evaluate the melting curves specific for *A. butzleri*. The temperature ramp was programmed from 65 °C to 90 °C, with increments of 0.5 °C for 5 sec. Samples were considered positive if both an exponential increase of fluorescence and the control-specific melting peak were observed.

Detection limit of SYBR Green real-time PCR

Serial concentrations of the reference bacterial solutions and pork-spike matrices were prepared using *A. butzleri* DMST19680. Briefly, one loop full of frozen stored *A. butzleri* DMST19680 was inoculated into 3 mL of AEB–CAT and incubated aerobically at 30°C for 48 h. Bacterial suspensions of 10-fold serial dilutions were made by transferring 1 mL of the enriched bacteria to the 9 mL AEB to make a 1:10 dilution, ranging from 10^{-1} to 10^{-9}. One mL of each 10-fold serial bacterial stock was seeded into 9 g of *Arcobacter*-negative pork homogenate, 9 bags for mixing with nine serial dilutions of the bacterial stock, and one bag for no inoculation (negative control). The serial dilutions of bacterial stock and pork-spike bacterial suspensions were used for the viable bacterial count, DNA extraction, and detection limit evaluation. Steps for SYBR Green real-time PCR optimization and detection limit are summarized in Figure 3. Enumerations of viable bacteria in the reference bacterial solutions and in the pork–spike matrices were performed on BA plates. Briefly, 1 mL from each dilution was transferred into a 9 mL AEB tube to make 10^{-1} to 10^{-9} concentrations of the original bag/tube. Afterward, 100 µL of each dilution was spread on BA plates and incubated at 30°C for 48 h under aerobic conditions. Colony forming unit (CFU) of *Arcobacter* per mL was calculated from the culture plates.

Validation and agreement between the two methods

Positive and negative results of *A. butzleri* in pork samples were compared between SYBR Green real-time PCR and bacterial culture-based methods. Sensitivity, specificity, and accuracy of SYBR Green real-time PCR were calculated using the 2 × 2 contingency table compared with the standard method, bacterial culture plus PCRs of 48 h PE samples. Cohen's kappa (*K*) statistic was used to evaluate the agreement between the two assays (Wang *et al*., 2019). Cohen's kappa index value (*K*) was between 0 and 1. Interpretation of agreement using *K* value followed a previous suggestion (Warrens, 2014). A large kappa probability (Z) value reflects reliability of the test or a high agreement between the two methods.

Results

Optimization and detection limit of SYBR Green real-time PCR

A. butzleri DMST19680 melting curves revealed a clear peak at 86.5°C (Figure 4). Therefore, positive *A. butzleri* in pork samples in the SYBR Green real-time PCR was considered under these conditions (Figure 4). The detection limit of SYBR Green real-time PCR in the reference *A. butzleri* DMST19680 solution was 2.8 x 10 CFU/mL, and in the pork-matrix was 2.8 × 10^4 CFU/mL (Figure 5).

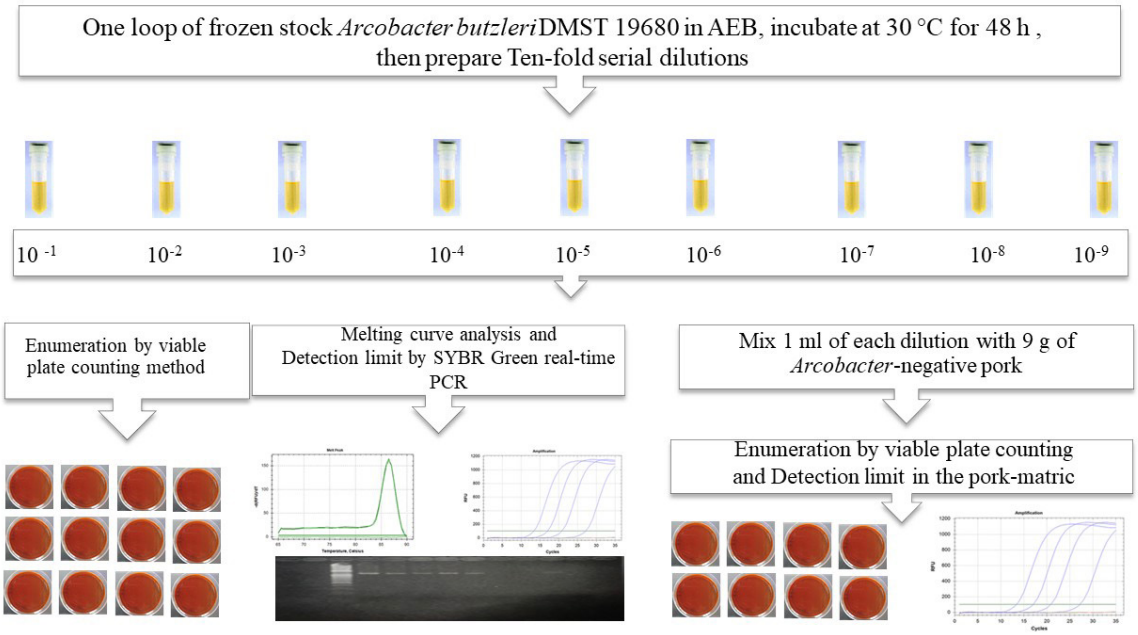

Figure 3. SYBR Green real-time PCR optimization and detection limit evaluation using reference serial concentrations of *A. butzleri* DMST19680.

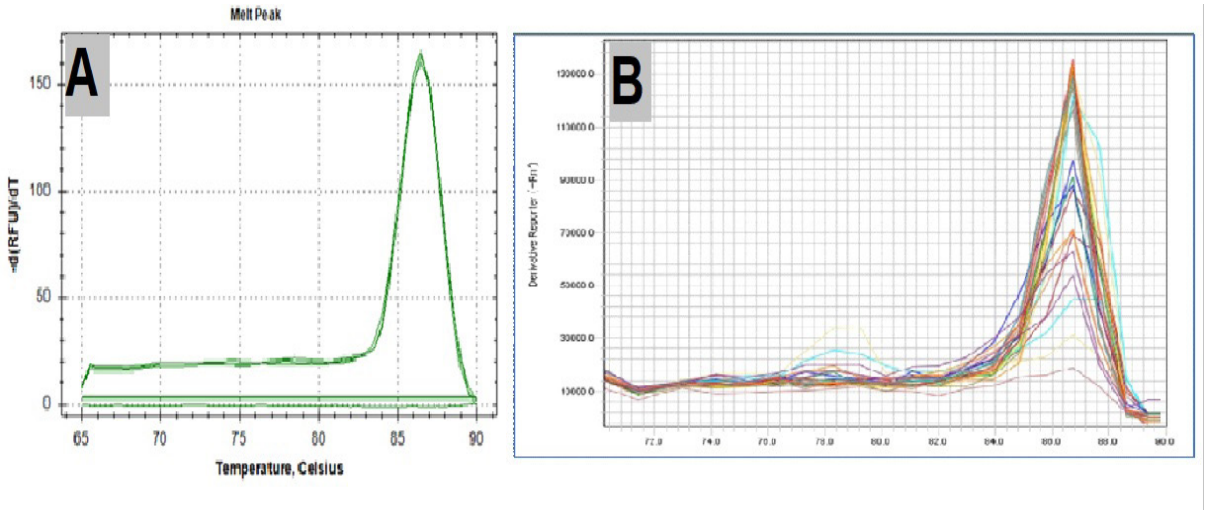

Figure 4. Melting curve analysis for *A. butzleri* reveals a clear peak at 86.5°C (A), so SYBR Green real-time PCR for *A. butzleri* detection in pork samples was considered at this point (B).

Occurrence of *Arcobacter butzleri* in pork samples

Presumptive *Arcobacter* colonies in bacterial culture were genus and species confirmed by classical PCR assays. The gold standard in this study was bacterial culture plus PCRs with 48 h PE. Among 60 pork samples of 48 h PE, 51 samples were *A. butzleri*-positive (85.0%) by the traditional method, whereas 54 samples were (90.0%) when using the real-time PCR approach. The bacterial culture-based diagnostic method detected *A. butzleri* in 2 (3.3%), 38 (63.3%), and 51 (85.0%) samples when the pork samples were pre-enriched for 0, 24, and 48 h, respectively. However, without PE (0 h PE) of samples, the SYBR Green real-time PCR result was negative in all 60 samples, whereas 54 (90.0%) *A. butzleri*-positive samples were found after 24 h and 48 h PE (Table 2).

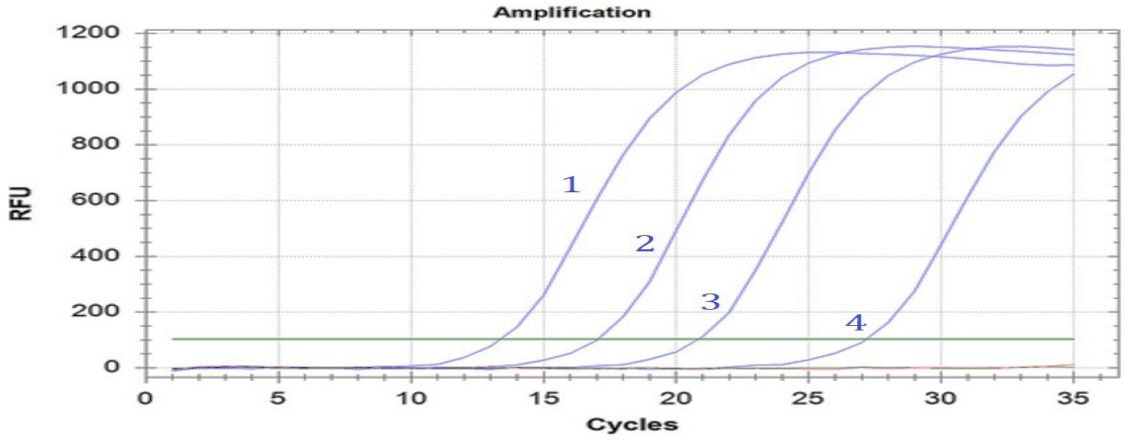

Figure 5. Amplification of species-specific 16S rRNA genes in the SYBR Green real-time PCR using serial 10-fold dilutions of *A. butzleri* DMST19680. Line 1 = 10^3, Line 2 = 10^2, Line 3 = 10^1, Line 4 = 10^0 CFU/mL, and red line = negative result.

Table 2. Positive proportion of *A. butzleri* in pork samples (*n* = 60) detected by SYBR Green real-time PCR and bacterial culture plus PCRs methods with 3 pre-enrichment periods before the assays

	Number of *A. butzleri* positive samples (%)	
Pre-enrichment period (hour)	SYBR Green real-time PCR	Bacterial culture plus PCRs
0	0 (0.0)	2 (3.3)
24	54 (90.0)	38 (63.3)
48	54 (90.0)	51 (85.0)

Validity and agreement between the two methods

The SYBR Green real-time PCR result was negative in all samples when pork samples were directly detected (0 h PE); thus, agreement between the two methods was not calculated for this set. As a shortened identification time was the goal, bacterial culture plus PCRs of 48 h PE were considered the reference method, and they were compared with 24 h PE in the SYBR Green real-time PCR assay. Binary results standing for 24 h PE of the SYBR Green real-time PCR and 48 h PE of the bacterial culture-based method are displayed in Table 3 to compute the sensitivity, specificity, accuracy, *K*, and *Z* values. Positive proportions of *A. butzleri* in samples detected by SYBR Green real-time PCR in 24 h PE and 48 h PE were the same (90.0%). The number of agreements and agreement by chance values were calculated (Table 3). The percentage of agreement was 95.0% (57/60), but this value included agreement by chance. The bacterial culture positive result was 85.0% (51/60), and the SYBER Green real-time PCR positive result was 90.0% (54/60). The positive agreement about these two diagnostic methods was due to chance, that is, 45.9 (85%*90%*60) of the cases. Similarly, negative agreement by chance of both tests was 0.9 (15%*10%*60), which means that 46.8 (45.9 + 0.9) of the diagnoses were due to chance. Then subtracting the agreement due to chance, this study obtained an agreement of 77.3%, which corresponded to Cohen's kappa (*K*) of 77.3% [(57 – 46.8)/(60 – 46.8)] in this study (Table 3).

Using bacterial culture as the gold reference, the sensitivity of SYBR Green real-time PCR was 100% in pork samples with 24 h and 48 h PE (Table 4). However, SYBR Green real-time PCR had a specificity of 27.3% (95%CI: 8.7–45.9) and 66.7% (95%CI: 35.9–97.5) and an accuracy of 73.3% (95%CI: 62.2–84.4) and 95% (95%CI: 89.5–100) with 24 h and 48 h PE periods, respectively (Table 4). Considering 48 h PE in the bacterial culture based as the reference method, SYBR Green real-time PCR with 24 h PE time had high sensitivity (100%) and high accuracy (95.0%;

Table 3. Agreement of the results by two methods calculated from number of positive and negative of SYBR Green real-time PCR assay (24 h PE) and bacterial culture plus PCRs with 48 h PE.

SYBR Green real-time PCR (24 h PE)	Bacterial culture plus PCR (48 h PE)		Total
	Number of positive	Number of negative	
Positive samples	51 (a)	3 (c)	54 (90.0%)
Negative samples	0 (b)	6 (d)	6 (10.0%)
Total	51 (85.0%)	9 (15.0%)	60 (n)
Calculated agreement			
Agreement of the result	51 (a)	6 (d)	57 (95.0%)
Agreement by chance	45.9	0.9	46.8

Note: PE = Pre-enrichment period, *K* = Cohen's kappa, n = total number of samples
Sensitivity = Number of true positive (a) / Total positive samples from conventional method (a + b)
Specificity = Number of true negative (d) / Total negative samples from conventional method (c + d)
Accuracy = (a + d) / (a + d + b + c)
positive agreement of the result = 57/60 = 95.0%
Positive agreement by chance = 85%*90%*n = 45.9
Negative agreement by chance = 15%*10%*n = 0.9
Total agreement by chance = 45.9 + 0.9 = 46.8
K = (number of positive by both tests – agreement by chance)/ (n- agreement by chance) = (57-46.8)/ (60-46.8) = 77.3%

Table 4. Sensitivity, specificity, accuracy, and Kappa co-efficient of SYBR Green real-time PCR assay compared to bacterial culture plus PCRs with 24 h or 48 h PE.

Assay versus Gold reference	SYBR Green real-time PCR versus Bacterial culture plus PCR		SYBR Green real-time PCR (24 h PE) versus Bacterial culture plus PCR (48 h PE)
Pre-enrichment	24 h PE	48 h PE	-
Positive samples	90.0% (95%CI: 82.4 - 97.6)	90.0% (95%CI: 82.4 - 97.6)	90.0% (95%CI: 82.4 - 97.6)
Sensitivity	100%	100%	100%
Specificity	27.3% (95%CI: 8.7– 45.9)	66.7% (95%CI: 35.9– 97.5)	66.7% (95%CI: 35.9 – 97.5)
Accuracy	73.3% (95%CI: 62.2 – 84.4)	95.0% (95%CI: 89.5– 100)	95.0% (95%CI: 89.5– 100)
K	32.2% (95%CI: 20.2– 44.2)	77.3% (95%CI: 59.7– 94.9)	77.3% (95%CI: 59.7– 94.9)
SE(k)	0.06	0.09	0.09
Z	5.37	8.59	8.59
Interpretation of K	Fair	Good	Good
Alpha	0.05	0.05	0.05
Z- critical value	1.96	1.96	1.96

Note: SE(*k*) = Reference error of kappa, 95%CI = 95% Confidence interval, *K* = Cohen's kappa, Z = Kappa probability
Sensitivity = Number of true positive (a) /Total positive samples from conventional method (a + b)
Specificity = Number of true negative (d) / Total negative samples from conventional method (c + d)
Accuracy = (a + d) / (a + d + b + c)
K = 2 (ad-bc) / (a+b) *(b+d) +(a+c) *(c+d)
Z = k / SE(*k*)

95%CI: 89.5–100) but relatively low specificity (66.7%; 95%CI: 35.9–97.5).

Discussion

Based on the results found in this study, a high prevalence of *A. butzleri* in pork meat was obtained in accordance with other reports (Kim et al., 2019; Vicente-Martins et al., 2018). Although other *Arcobacter* species have been found in pork meat, this study focused solely on *A. butzleri*. According to other studies, *A. butzleri* is the most common species of *Arcobacter* found in foods and animals. A study in Spain reported that *Arcobacter* was detected in 53.0% of pork samples; *A. butzleri* was the main species, with small frequencies of *A. skirrowii* (Collado *et al*., 2009). In addition, a survey in Brazil isolated *A. butzleri* as the most frequent species, followed by *A. cryaerophilus*, in pork meat (Gobbi *et al*., 2018). Similarly, *A. butzleri* and *A. skirrowii* were the only two species present in pork samples in Korea (Kim *et al*., 2019). Nevertheless, species differences may be due to the chronology, geography, or media used in bacterial isolation. In a previous study, it was noted that using mCCDA agar yielded higher recovery rates than *Arcobacter* selective agar (Kim *et al*., 2019). Furthermore, selective PE broth itself may alter the detection of *Arcobacter* species in food samples. For example, direct plating identified *A. cryaerophilus* instead of *A. butzleri*, but opposite results were found after PE of wastewater samples before plating (Levican *et al*., 2016). Therefore, selective PE could favor the recovery of *A. butzleri* in the samples. Unfortunately, in the current study, only two samples were *Arcobacter* genus positive by direct plating (0 h PE), and they were identified as *A. butzleri* by classical PCR.

The results showed that SYBR Green real-time PCR was sensitive and specific. However, the performance of SYBR Green real-time PCR to detect *Arcobacter* was likely compromised in food samples. Because the sensitivity of the test was reduced in the pork matrices, or in other words, 1,000 X bacterial numbers were needed to be positive (detection limit) as compared with pure bacterial solutions. Given that a small amount of *Arcobacter* (beyond the detection limit) was present in pork samples, PE and culture media could expand the live numbers of *Arcobacter*. At this point, SYBR Green real-time PCR did not detect *Arcobacter* in pork meat directly. This was most likely due to the presence of low bacterial quantities that were beyond the test's detection ability. Selective PE may increase the number of *Arcobacter* and inhibit other bacteria in food samples. Regarding bacterial culture-based identification, direct plating yielded only 87.6%, which increased to 100% after samples were pre-enriched before the test (Levican *et al*., 2016). In contrast to our results, a former study found that direct molecular detection (10/24) yielded a slightly higher number (one more sample) of *Arcobacter*-positive pork samples than bacterial isolation (9/24) (Vicente-Martins et al., 2018). In applying SYBR Green real-time PCR with selective 48-h PE to chicken samples, *Arcobacter* was found to be 80.5% (González et al., 2010) and 56% (Ramees *et al*., 2014). In the present study, a remarkably high prevalence (90.0%) of *Arcobacter* was found in pork samples with 24 h and 48 h PE periods. The results show that the real-time PCR method can get results in a shorter period than traditional identification. It is important to note, however, that pre-enrichment of pork increases detection rates despite the low concentration of bacteria in a sample. And this results in a high frequency of *Arcobacter*-positive pork. In general, real-time PCR is more sensitive and specific as compared with the bacterial culture method. Considering this, SYBR Green real-time PCR produced low specificity when compared to bacterial isolation as the gold reference. SYBR Green real-time PCR detected *A. butzleri* in pork samples at the same rate between 24 h and 48 h PE. The *K* value of 77.3% (95%CI: 59.7-94.9) between bacterial cultures plus PCRs (48 h PE) and SYBR Green real-time PCR (24 h PE) assays is regarded as a good agreement. This finding suggests that 24 h PE in SYBR Green real-time PCR on pork samples is a reliable diagnostic method for *A. butzleri* detection in pork.

Conclusions

A. butzleri is highly prevalent in pork samples sold in Mueang district, Khon Kaen, Thailand. In detecting *A. butzleri* in pork samples, bacterial culture based and SYBR Green real-time PCR assays agreed well. Pre-enrichment is still needed, however, to diagnose low levels of *Arcobacter* contamination in pork samples that exceed the defection limit. SYBR Green real-time PCR needs a shorter period (2–3 days), but bacterial culture-based identification needs 5-8 days for *Arcobacter* diagnosis. SYBR Green real-time PCR with 24 h PE, in conclusion, is a reliable screening method for *Arcobacter* detection in pork samples.

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References

Aydin F, Yağiz A, Abay S, Müştak HK, Diker KS, 2020. Prevalence of *Arcobacter* and *Campylobacter* in beef meat samples and characterization of the recovered isolates. J fur Verbraucherschutz und Leb 15, 15–25.

Bodhidatta L, Srijan A, Serichantalergs O, Bangtrakulnonth A, Wongstitwilairung B, McDaniel P, Mason CJ, 2013. Bacterial pathogens isolated from raw meat and poultry compared with pathogens isolated from children in the same area of rural Thailand. Southeast Asian J Trop Med Public Health 44, 259.

Brückner V, Fiebiger U, Ignatius R, Friesen J, Eisenblätter M, Höck M, Alter T, Bereswill S, Heimesaat MM, Gölz G, 2020. Characterization of *Arcobacter* strains isolated from human stool samples: Results from the prospective German prevalence study Arcopath. Gut Pathogens. https://doi.org/10.1186/s13099-019-0344-3.

Bujang MA, Adnan TH, 2016. Requirements for minimum sample size for sensitivity and specificity analysis. J Clin Diagnostic Res 10, YE01–YE06. https://doi.org/10.7860/JCDR/2016/18129.8744.

Collado L, Guarro J, Figueras MJ, 2009. Prevalence of *Arcobacter* in meat and shellfish. J Food Prot 72, 1102–1106.

Fernandez H, Villanueva MP, Mansilla I, Gonzalez M, Latif F, 2015. *Arcobacter butzleri* and *A. cryaerophilus* in human, animals and food sources, in southern Chile. Brazilian J Microbiol 46. https://doi.org/10.1590/S1517-838246120140095.

Ferreira S, Oleastro M, Domingues F, 2019. Current insights on *Arcobacter butzleri* in food chain. Curr Opin Food Sci 26, 9–17.

Gobbi DDS, Spindola MG, Moreno LZ, Matajira CEC, Oliveira MGX, Paixão R, Ferreira TSP, Moreno AM, 2018. Isolation and molecular characterization of *Arcobacter butzleri* and *Arcobacter cryaerophilus* from the pork production chain in Brazil. Pesqui Vet Bras 38, 393–399.

González A, Bayas Morejón IF, Ferrús MA, 2017. Isolation, molecular identification and quinolone-susceptibility testing of *Arcobacter* spp. isolated from fresh vegetables in Spain. Food Microbiol 65, 279–283.

González A, Suski J, Ferrús MA, 2010. Rapid and accurate detection of *Arcobacter* contamination in commercial chicken products and wastewater samples by real-time polymerase chain reaction. Foodborne Pathog Dis 7. https://doi.org/10.1089/fpd.2009.0368.

González I, Fernández-Tomé S, García T, Martín R, 2014. Genus-specific PCR assay for screening *Arcobacter* spp. in chicken meat. J Sci Food Agric 94. https://doi.org/10.1002/jsfa.6401.

Houf K, Tutenel A, Zutter L, Hoof J, Vandamme P, 2000. Development of a multiplex PCR assay for the simultaneous detection and identification of *Arcobacter butzleri*, *Arcobacter cryaerophilus* and *Arcobacter skirrowii*. FEMS Microbiol Lett 193, 89–94.

Jiménez-Guerra G, Moreno-Torres IC, Moldovan TD, Navarro-Marí JM, Gutiérrez-Fernández J, 2020. *Arcobacter butzleri* and intestinal colonization. Rev Esp Qimioterapia 33, 73–75.

Kim NH, Park SM, Kim HW, Cho TJ, Kim SH, Choi C, Rhee MS, 2019. Prevalence of pathogenic *Arcobacter* species in South Korea: Comparison of two protocols for isolating the bacteria from foods and examination of nine putative virulence genes. Food Microbiol 78, 18–24.

Levican A, Collado L, Figueras MJ, 2016. The use of two culturing methods in parallel reveals a high prevalence and diversity of *Arcobacter* spp. in a wastewater treatment plant. Biomed Res Int 2016. https://doi.org/10.1155/2016/8132058.

Mizutani Y, Iehata S, Mori T, Oh R, Fukuzaki S, Tanaka R, 2019. Diversity, enumeration, and isolation of *Arcobacter* spp. in the giant abalone, Haliotis gigantea. Microbiologyopen 8, 1–10.

Morejón IFB, González A, Ferrús MA, 2017. Detection, identification, and antimicrobial susceptibility of *Arcobacter* spp. isolated from shellfish in Spain. Foodborne Pathog Dis 14, 238–243.

Morita Y, Maruyama S, Kabeya H, Boonmar S, Nimsuphan B, Nagai A, Kozawa K, Nakajima T, Mikami T, Kimura H, 2004. Isolation and phylogenetic analysis of *Arcobacter* spp. in ground chicken meat and environmental water in Japan and Thailand. Microbiol Immunol 48, 527–533.

Niedermeyer JA, Miller WG, Yee E, Harris A, Emanuel RE, Jass T, Nelson N, Kathariou S, 2020. Search for *Campylobacter* spp. Reveals high prevalence and pronounced genetic diversity of *Arcobacter butzleri* in floodwater samples associated with hurricane florence in North Carolina, USA. Appl. Environ Microbiol 86. https://doi.org/10.1128/AEM.01118-20.

Ramees TP, Dhama K, Karthik K, Rathore RS, Kumar A, Saminathan M, Tiwari R, Malik YS, Singh RK, 2017. *Arcobacter*: An emerging food-borne zoonotic pathogen, its public

health concerns and advances in diagnosis and control - A comprehensive review. Vet Q 37, 136–161.

Ramees TP, Rathore RS, Bagalkot PS, Kumar GVPPSR, Mohan HV, Anoopraj R, Kumar A, Dhama K, 2014. Real-time PCR detection of *Arcobacter butzleri* and *Arcobacter cryaerophilus* in chicken meat samples. J Pure Appl Microbiol 8, 3165–3169.

Sekhar MS, Tumati SR, Chinnam BK, Kothapalli VS, Sharif NM, 2017. Virulence gene profiles of Arcobacter species isolated from animals, foods of animal origin, and humans in Andhra Pradesh, India. Vet World 10, 716–720.

Shrestha RG, Tanaka Y, Sherchand JB, Haramoto E, 2019. Identification of 16s rRNA and virulence-associated genes of *Arcobacter* in water samples in the Kathmandu valley, Nepal. Pathogens 8, 1–9.

Soma Sekhar M, Tumati SR, Chinnam BK, Kothapalli VS, Sharif NM, 2018. Occurrence of *Arcobacter* species in animal faeces, foods of animal origin and humans in Andhra Pradesh, India. Indian J Anim Res 52, 1649–1653.

Teague NS, Srijan A, Wongstitwilairoong B, Poramathikul K, Champathai T, Ruksasiri S, Pavlin J, Mason CJ, 2010. Enteric pathogen sampling of tourist restaurants in Bangkok, Thailand. J Travel Med 17. https://doi.org/10.1111/j.1708-8305.2009.00388.x.

Tomioka N, Yoochatchaval W, Takemura Y, Matsuura N, Danshita T, Srisang P, Mungjomklang N, Syutsubo K, 2021. Detection of potentially pathogenic *Arcobacter* spp. in Bangkok canals and the Chao Phraya River. J Water Health 19, 657–670.

Vicente-Martins S, Oleastro M, Domingues FC, Ferreira S, 2018. *Arcobacter* spp. at retail food from Portugal: Prevalence, genotyping and antibiotics resistance. Food Control 85, 107–112.

Vindigni SM, Srijan A, Wongstitwilairoong B, Marcus R, Meek J, Riley PL, Mason C, 2007. Prevalence of foodborne microorganisms in retail foods in Thailand. Foodborne Pathog Dis 4. https://doi.org/10.1089/fpd.2006.0077.

Wang J, Yang Y, Xia B, 2019. A simplified Cohen's Kappa for use in binary classification data annotation tasks. IEEE Access 7. https://doi.org/10.1109/ACCESS.2019.2953104.

Warrens MJ, 2014. New interpretations of cohen's kappa. J Math 2014. https://doi.org/10.1155/2014/203907.

Webb AL, Boras VF, Kruczkiewicz P, Selinger LB, Taboada EN, Inglis GD, 2016. Comparative detection and quantification of *Arcobacter butzleri* in stools from diarrheic and nondiarrheic people in Southwestern Alberta, Canada J Clin Microbiol 54. https://doi.org/10.1128/JCM.03202-15.

Zhang X, Alter T, Gölz G, 2019. Characterization of *Arcobacter* spp. isolated from retail seafood in Germany. Food Microbiol 82. https://doi.org/10.1016/j.fm.2019.02.010.