Journal of Microbiological Methods 157 (2019) 117–122

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Development and evaluation of a novel *Campylobacter* spp. enrichment medium

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ARTICLE INFO

Keywords:
Campylobacter
Enrichment medium
Food matrices

ABSTRACT

Although rapid detection kits continue to be developed and used, the classical culture method is still accepted as a gold standard. Therefore accelerating the classical culture methods safely is important for the detection of *Campylobacter*. The aim of this study is to design and compare a novel developed medium with other confirmed media in naturally and experimentally contaminated food matrices. Besides classical culture methods, it is subjected to qPCR and FISH methods. In this study, *Campylobacter* counts are investigated in spiked milk, chicken breast meat, cumin, minced beef meat, celery and tomato puree. Also to evaluate the enrichment medium in naturally contaminated samples, *Campylobacter* detection is performed in 20 chicken neck skin samples obtained from different sales points. The study showed that the novel broth provides a faster detectable number of *Campylobacter*. It was found to provide detectable *Campylobacter* counts after eight hours of inoculation. The results have shown that there is a significant increase on *Campylobacter* count in the detection performed using the spiked foods. Furthermore, the entire natural contaminated chicken neck skin samples are found to be positive the same as the other mediums. As a result of the study, in the classic culture methods, designed enrichment medium is faster than the currently used enrichment mediums. It is an important point of view to develop fast and reliable diagnostic methods for assuring adequate public health.

1. Introduction

Campylobacter is a gram-negative, spiral-shaped, motile, microaerophilic food-borne pathogen and over 90% cases of food-borne campylobacteriosis were caused by thermotolerant groups including *Campylobacter jejuni* (Bolton, 2015; Griffiths and Park, 1990). *Campylobacter* spp. are found commensally in the intestinal system of warm-blooded animals such as cattle, sheep, pigs and poultry. Due to their environmentally wide distribution, animal originated foods poses infection threats (Baylis et al., 2000). Campylobacteriosis is the most reported food-borne illness annually in the European Union since 2005. According to the EFSA (European Food Safety Authority), 246,307 cases of verified human campylobacteriosis have been reported in 2016 and the number of cases has increased by 6.1% compared to 2015 (EFSA, 2017).

Campylobacter isolation has to be more accurate than the other food-borne pathogens due to its high sensitivity to oxygen and oxidizing radicals, water activity and temperature changes and presence of viable-but-nonculturable forms (VBNC) (Portner et al., 2007; Silva et al., 2011). Although *C. jejuni* is so fragile, campylobacteriosis is one of the most common food-borne infections due to its minimum infectious dose that is estimated to be in between 500 and 800 microorganisms (Black et al., 1988).

Standardized protocols have been developed by organizations such as ISO (International Standardization Organization) and FDA (U·S Food and Drug Administration) and various media have been developed recently and various studies for recovery also have been carried out to reduce the time of detection and increasing the sensitivity (Abay et al., 2014; Hunt et al., 2001; ISO, 2006).

Rapid detection kits continue to be developed and used; the classical culture method is still accepted as a gold standard. Therefore, decreasing the incubation time in the classical culture method is important in the detection of *Campylobacter*. The classical *Campylobacter* detection method is generally designed as plating onto specific agar after selective enrichment step. Nowadays, Bolton Broth (BB) and Preston Broth (PB) are commercially available and widely used in the

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https://doi.org/10.1016/j.mimet.2019.01.004
Received 30 November 2018; Received in revised form 3 January 2019; Accepted 11 January 2019
Available online 11 January 2019
0167-7012/

detection of *Campylobacter* as enrichment medium. Principally these media are used to recover sub-lethal damaged cells. The aim of this study was to determine the effectiveness of novel prepared enrichment medium (ASK Broth). For this purpose; the following was conducted to (i) compare validated selective enrichment media; (ii) *Campylobacter* detection in six different food matrices; and (iii) detection of the presence of *Campylobacter* spp. in the natural contaminated chicken neck skin samples.

2. Materials and methods

2.1. Strains

Seven different wild-type *C. jejuni* strains (isolated from two chicken neck skin, chicken breast meat, chicken feces, turkey meat and two milk samples) were used in classical culture, PCR and FISH methods. In addition, *C. jejuni* ATCC 33560, *C. coli* ATCC 33559 and *E. coli* ATCC 25922 strains were used as positive and negative controls in Fluorescence in situ hybridization (FISH) and Quantitative Polymerase Chain Reaction (qPCR) analyzes.

2.2. Preparation and Content of the New developed Enrichment Medium (ASK Broth)

The content of the newly developed medium is given in Table 1. All content except lysed horse blood and antibiotics were added to 1.0 L sterile distilled water and then it then autoclaved. After sterilization, media were cooled to 50 ± 1 °C and supplements were added. The final pH of the medium was measured as 7.8 and the prepared liquid medium stored at +4 °C before use.

2.3. Determination of the effectiveness of ASK enrichment medium and comparison with others

International Organization for Standardization (ISO) 10272-1 standard detection protocol was followed to compare the efficacy of ASK Broth, Bolton broth (Merck, 100068) with Bolton Broth Selective supplement (Merck, 100079) and Preston broth (Oxoid, CM0067) with Preston *Campylobacter* Selective Supplement (Oxoid, SR0117). Wild type *C. jejuni* strains were comparatively examined in the classical culture, Fluorescence in situ hybridization (FISH) and Quantitative Polymerase Chain Reaction (PCR) methods.

Table 1
Content of the novel developed enrichment medium (ASK Broth).

Content	Amount per liter
Peptone from casein	5.0 g
Papaic digest of soyabean meal	1.0 g
Beef extract	5.0 g
Yeast extract	2.0 g
DL-serine	3.0 g
DL-proline	3.0 g
Na-acetate	1.0 g
Oxaloacetic acid	0.1 g
Di-sodium succinate	1.0 g
Ferrous fumarate	1.0 g
Ammonium molybdate tetrahydrate	0.1 g
Magnesium tungstate	0.1 g
Ferritin	0.01 g
Na bicarbonate	0.1 g
Lysed horse blood	10 mL
2500 IU polymixin B	5.0 mg
Rifampicin,	5.0 mg
Trimethoprimlactate	50 mg
Cycloheximide	50 mg in 5.0 mL acetone

2.3.1. Classical culture method

Seven wild type *C. jejuni* strains used in the study were inoculated in three different media (ASK, BB and PB) and incubated in a microaerophilic conditions (7% O_2, 10% CO_2, 83% N_2) at 41,5 °C. At 6, 8, 12 and 24 h of incubation period, inoculums were plated onto Modified Charcoal Cefoperazone Deoxycholate Agar (Merck, 100,070) with CCDA Selective Supplement (Merck, 100,071) to determine the enrichment efficiency of broths by the time.

2.3.2. qPCR conditions

For *Campylobacter jejuni* detection, inoculated enrichment media were subjected to BAX® System Real-Time PCR Assay for *Campylobacter* ready test kit (Hygiena, USA) according to the manufacturer's instructions. qPCR was performed with the Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument (Thermo, Finland).

2.3.3. Fluorescence in situ hybridization

In the study, Vermicon AG VIT® *Campylobacter* test kits were used according to the manufacturer's instructions. The samples visualized by Nikon Eclipse 80i with filters B2e/C Fluorescein isothiocyanate (FITC).

2.4. Campylobacter detection in spiked food samples

To reveal the effectiveness of ASK Broth in *C. jejuni* detection from various food samples, 10 mL milk, 10 g of chicken breast meat, cumin, minced beef meat, celery and tomato puree samples were used. Autoclaved (15 min at 121 °C) food samples were rehydrated with sterile water to substitute the weight loss. For spiking, an overnight fresh culture of *C. jejuni* ATCC 33560 was inoculated (2.70 $\log_{10}$ cfu/ml) with sterilized food samples and changes in *Campylobacter* counts were determined at 2, 4, 6, 8, 12, and 24 h of incubation period.

2.5. Detection of the presence of campylobacter spp. in the natural contaminated chicken neck skin samples

A total of 20 chicken neck samples were collected and the presence of *Campylobacter jejuni* was investigated with the classical culture method, qPCR and FISH methods in the 8, 12 and 24 h of enrichment period. ASK Broth and PB were cooperatively used in the enrichment step of *Campylobacter* detection protocols.

2.6. Statistical analysis

The effects of used enrichment media, analyzing time and detection methods on *Campylobacter* detection percentages were evaluated by Chi-squared Automatic Interaction Detector Analysis (CHAID). CHAID analysis based on a dependent variable (*Campylobacter* detection percentages) and independent variables (enrichment media, analyzing time and detection methods) allows determining the segmentation of variable and the suitable combination of a range of independent variables via Chi-Square analyses.

In the evaluation of the enrichment capability of ASK medium in spiked foods, the statistical significance of the difference by the time was determined by Friedman's Two-Way Analysis of Variance. Also, detection rates of *Campylobacter* in the natural contaminated chicken neck skin samples between ASK Broth and PB were compared with chi-square tests. Microbiological analyses in the study were carried out in duplicate and mean data were expressed as $\log_{10}$ cfu/g. All statistical analysis in the study were performed with SPSS 14.01 package program.

Table 2
Comparison of the enrichment effectiveness of ASK, PB and BB according to incubation time with different detection methods.

	6 h			8 h			12 h			24 h		
	FISH	qPCR	CCM	FISH	qPCR	CCM	FISH	qPCR	CCM	FISH	qPCR	CCM
Detection performances using ASK Broth with different detection methods												
Strain A	+	+	−	+	+	+	+	+	+	+	+	+
Strain B	+	+	−	+	+	+	+	+	+	+	+	+
Strain C	+	+	−	+	+	+	+	+	+	+	+	+
Strain D	+	−	+	+	+	+	+	+	+	+	+	+
Strain E	+	+	−	+	+	+	+	+	+	+	+	+
Strain F	+	+	+	+	+	+	+	+	+	+	+	+
Strain G	+	+	+	+	+	−	+	+	+	+	+	+
Detection performances using Preston Broth with different detection methods												
Strain A	+	+	−	+	+	−	+	+	−	+	+	+
Strain B	+	−	−	+	+	−	+	+	−	+	+	+
Strain C	−	+	−	−	−	−	+	+	−	+	+	+
Strain D	+	−	−	+	+	+	+	+	+	+	+	+
Strain E	−	+	−	+	+	−	+	+	−	+	+	+
Strain F	+	+	+	+	+	+	+	+	+	+	+	+
Strain G	+	−	−	+	+	+	+	+	+	+	+	+
Detection performances using Bolton Broth with different detection methods												
Strain A	−	−	−	+	+	−	+	+	−	+	+	+
Strain B	+	−	−	+	+	−	+	+	−	+	+	+
Strain C	−	−	−	−	−	−	+	+	+	+	+	+
Strain D	−	−	−	+	−	+	+	−	+	+	+	+
Strain E	−	+	−	−	+	−	−	+	−	+	+	+
Strain F	−	−	−	−	−	−	+	+	−	+	+	+
Strain G	−	+	−	+	+	+	+	+	+	+	+	+

+: Positive Result; −: Negative Result; FISH: Fluorescence in situ hybridization; qPCR: Quantitative Polymerase Chain Reaction; CCM: Classical Culture Method.
Strain A - *C.jejuni* from chicken neck skin.
Strain B - *C.jejuni* from turkey meat.
Strain C - *C.jejuni* from chicken neck skin.
Strain D - *C.jejuni* from milk.
Strain E - *C.jejuni* from chicken feces.
Strain F - *C.jejuni* from chicken breast.
Strain G - *C.jejuni* from milk.

3. Results

3.1. Determination and comparison of the effectiveness of ASK enrichment medium

Comparison of ASK Broth with BB and PB in the detection of seven different food origin *Campylobacter jejuni* isolates are shown in Table 2. Fig. 1 shows CHAID Analysis for evaluating the hierarchical effects of used enrichment media analyzing time and detection methods on the *Campylobacter* detection results, as well as FISH results are given in Fig. 2.

3.2. Campylobacter counts in experimental contaminated food samples

Campylobacter detection using ASK Broth in spiked food (milk, chicken breast meat, cumin, minced beef meat, celery and tomato puree) and recovery capability of ASK Broth are given in detail in Table 3 and Fig. 3. It was observed that in the enrichment with ASK Broth in *Campylobacter* isolation from various food samples, *Campylobacter* counts were increased approximately 3.2 $\log_{10}$ cfu/ml. It was determined that the linear increase of *Campylobacter* counts ($\log_{10}$ cfu/g) in all spiked food samples by the time were statistically significant ($p < .001$). Comparison of the spike levels, there were no significant differences between 2 and 4 h of detection, while the statistical difference was revealed at 6 h of the incubation period.

3.3. Campylobacter counts in natural contaminated chicken neck skin samples

The results of 20 naturally contaminated chicken neck skin samples were examined for *Campylobacter* detection using ASK and PB and the results are shown in Table 4. It was determined that after 24 h enrichment, all the chicken neck samples were positive for *Campylobacter* spp.

At the 8 and 12 h of incubation period, it was found that ASK Broth was more effective than PB for enrichment purposes. It was revealed that there were no significant difference between the enrichment efficiency of ASK and PB in FISH and qPCR methods at all analysis times ($p > .05$).

At the 8th hour of the incubation period it was detected that using classical culture methods with ASK Broth were 15 (75%) positive samples and using PB only 6 (30%) positive samples were detected. At the 12 h, 19 (95%) and 13 (65%) positive sample were detected in ASK Broth and PB, respectively. The differences between the enrichment efficiency of ASK broth and PB in the classical culture method at 8 and 12 h of the incubation period were statistically significant ($p < .01$). The results revealed that, at 8 and 12 h of enrichment of naturally contaminated neck skins, ASK broth yielded significantly more positive results than PB.

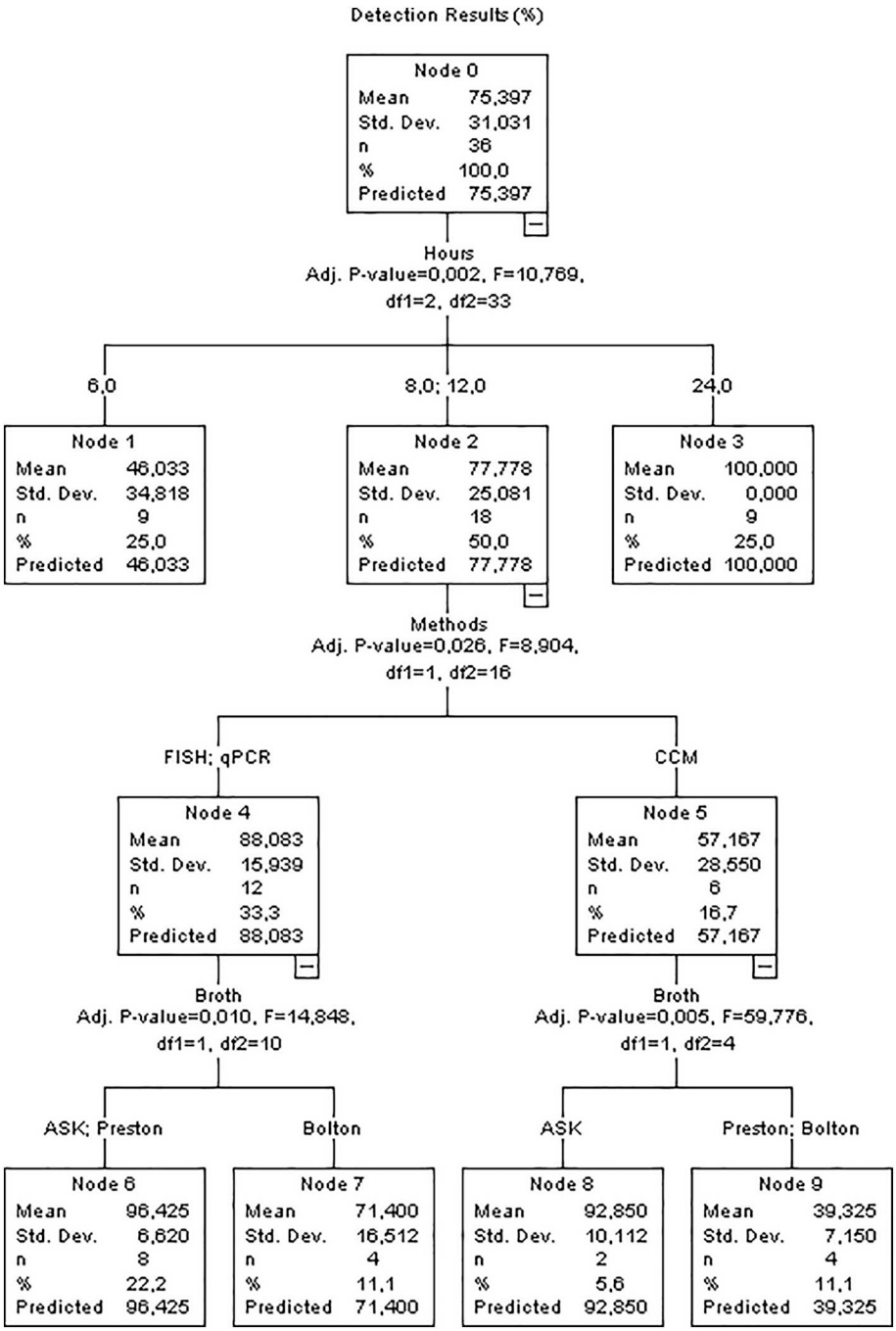

Fig. 1. Classification tree (Chaid growing method) of the *Campylobacter* detection percentages of ASK, PB and BB according to incubation time with different detection methods.

4. Discussion

Fast and reliable detection of food-borne pathogens are important for public health and the development of selective media for the isolation of fragile but highly pathogenic microorganisms from various samples is an active research area. Particularly, in the detection of lower counts of pathogens, enrichment steps are a very important tool for selective determination of pathogens in foods (Acke et al., 2009; Shin and Lee, 2009). Enrichment processes generally require 24 h or more incubation. Decreasing of incubation period is particularly important for prevention of outbreaks. In this way, food pathogens would be detected as soon as possible and epidemiological data of outbreaks can be revealed promptly. As part of the study, the novel developed broth (ASK), PB and BB, were compared with recovery and enrichment performances. As a result, it was revealed that the three broths provided sufficient growth at 24th hours, but the ASK Broth achieved a faster enrichment than the other two broths especially when using classical culture technique. In routine controls and food-borne outbreaks, it is epidemiologically essential to ensure rapid and reliable detection. For this purpose, it would be very useful to accelerate classical culture techniques which are accepted as the gold standard.

Despite that, BP is the currently recommended medium by the U.S. Food and Drug Administration (Hunt et al., 2001) and the International Standard Organization (ISO, 2006), some studies were revealed that BP showed a good compromise between recovery of *Campylobacter* and inhibition of competitive microorganisms such as *E. coli* and

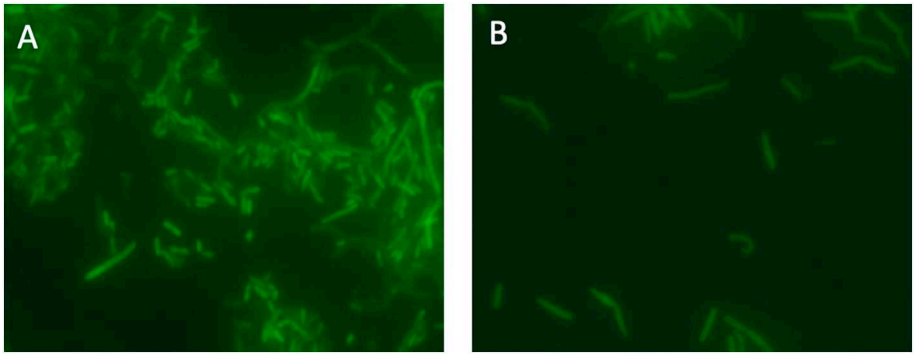

Fig. 2. Epifluorescence micrograph of *C. jejuni* by FISH detection method. A; *C. Jejuni* detected with the green fluorescein probe. B; *C. Jejuni* detected with the green fluorescein probe for living bacteria. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
C. jejuni ATCC 33560 counts in experimental contaminated (spiked) food samples using ASK broth according to incubation time (log_{10} cfu/g-mL).

Foods	Spike level	Incubation time (hour)						
		2	4	6	8	10	12	24
Milk	2,68	2,62	2,77	2,95	3,02	3,50	3,99	5,87
Chicken breast	2,67	2,69	2,73	2,99	3,32	3,93	4,83	6,46
Cumin	2,70	2,49	2,51	2,85	2,99	3,72	4,23	5,59
Minced beef meat	2,69	2,70	2,89	2,96	3,47	3,90	4,89	6,02
Celery	2,68	2,61	2,68	2,77	3,20	3,72	4,04	5,72
Tomato puree	2,66	2,31	2,61	2,84	2,94	3,44	3,74	5,62

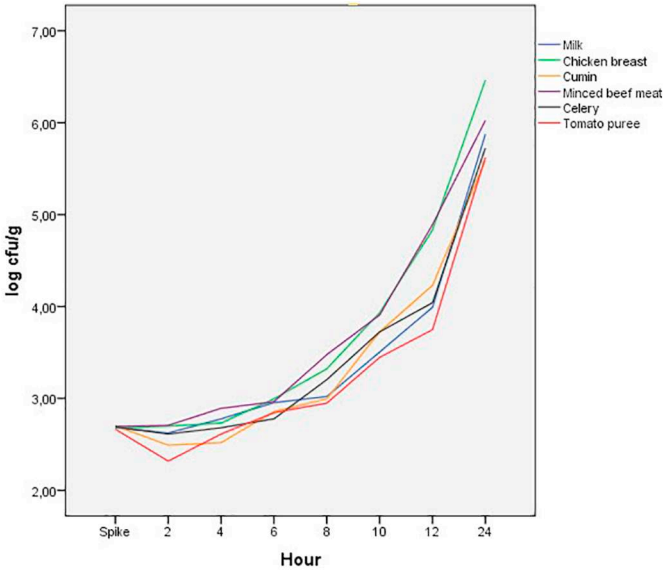

Fig. 3. Representative growth curve of *C. jejuni* ATCC 33560 in experimental contaminated (spiked) food samples in ASK broth according to incubation time.

Pseudomonas spp. (Baylis et al., 2000). In addition, it was shown that BP supported the growth of extended-spectrum-beta-lactamase (ESBL) *E. coli*; and lead to false-negative results (Jasson et al., 2009).

PB was determined as the good selective broth for *Campylobacter* detection (Bolton and Robertson, 1982; Habib et al., 2011; Jasson et al., 2009; Ugarte-Ruiz et al., 2012; Uyttendaele and Debevere, 1996). However, it was claimed that PB may inhibit growth of some *Campylobacter* strains and shows false negative results (Baylis et al., 2000; Paulsen et al., 2005). But, similar to the findings of this study, it was shown that good growth curves in PB recorded with both *C. jejuni* reference strains and wild type chicken originated from *Campylobacter* (Hazeleger et al., 2016).

According to the findings of the CHAID analyses, the most effective factor on the positivity rate was incubation time. At least 8 h of selective enrichment incubation was required to show any effect on the positivity rate of the applied detection method. It was indicated that the positive detection rates of FISH and qPCR methods were higher than classical culture methods (57%) at the 8 and 12 h of selective enrichment period. In the selective enrichment procedure performed for FISH and qPCR detection methods, the positivity rate of ASK and PB (96.42%) broth was higher than BP. In the analysis with classical culture methods, the positivity rate of ASK Broth (92.8%) enrichment was found to be higher than PB and BP (39.3%) (Fig. 1). As the main finding of the study; it is revealed that enrichment time can be reduced to 12 h by using ASK Broth, if the classical culture method is used in the *Campylobacter* detection.

In this study, different types of food samples such as milk, chicken breast meat, cumin, minced beef meat, celery and tomato puree were spiked with *Campylobacter*. It was determined that each of three broths used in the detection of *Campylobacter* in contaminated foods provided adequate enrichment. In particular, the novel developed broth (ASK) has been shown to provide enrichment as much as the currently used broths and the structure of food has no negative effect on the enrichment performance.

The enrichment performance of the broth developed in the study was also evaluated in naturally contaminated foods. For this purpose,

Table 4
Comparison of the *Campylobacter* spp. detection rates using ASK and PB according to incubation time with different detection methods in naturally contaminated chicken neck skin samples (n = 20).

	ASK Broth			Preston Broth		
	FISH	qPCR	CCM	FISH	qPCR	CCM
8 h	17 (%85)	18 (%90)	15 (%75)*	12 (%60)	16 (%80)	6 (%30)*
12 h	20 (%100)	20 (%100)	19 (%95)*	14 (%70)	17 (%85)	13 (%65)*
24 h	20 (%100)	20 (%100)	20 (%100)	20 (%100)	20 (%100)	20 (%100)

FISH: Fluorescence in situ hybridization; qPCR: Quantitative Polymerase Chain Reaction; CCM: Classical Culture Method.
* The differences of detection rates between ASK broth and PB in the CCM were statistically significant ($p < .01$).

Campylobacter detection was performed with PB of 20 chicken neck skin samples collected from different sales points. In the three detection analyses, it was found that both broths were successful at 24 h of the enrichment period, but ASK broth at 12 h showed much more effective enrichment performance ($p < .01$). Compatibility of this part of the study with other findings is very important for the confirmation of enrichment capability of the developed broth.

5. Conclusions

It was revealed that the three different enrichment broths showed different enrichment performance in the current study. Not only the media used but also the incubation time and the detection method is also effective on the enrichment process. In addition, factors such as type of food, level of contamination, competitive microbiota in food are effective in the detection of food-borne pathogens. The novel developed enrichment medium (ASK Broth) is not statistically different from other broths when DNA-based molecular technologies with high sensitivity are used. However, when it is needed to use the classical culture method; it was revealed that ASK broth shows faster results than BP and PB. As a results of this, it is recommended to use ASK enrichment broth when classical culture method is selected. Food-borne outbreaks can be limited and prevented with rapid diagnosis and detection. In this manner, this study showed that the gold standard method can be effectively decreased.

Disclosure statement

The authors declare that there is no conflict of interest in this study.

Acknowledgements

The abstract of this study was presented at The 3rd International Congress on Food Technology on 10-12 October 2018, Nevşehir-Turkey.

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