

Evaluation of a novel and rapid screening method for the detection of contaminated colistin-resistant bacteria in food

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Short Report

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Abstract

Background: The dissemination of colistin-resistant bacteria carrying the colistin-resistant mobile gene, *mcr-1* threatens medical care worldwide. In particular, contamination of food with colistin-resistant bacteria accelerates the community dissemination of colistin-resistant bacteria. Therefore, monitoring of colistin-resistant bacteria in food is important for controlling resistant bacteria. Unfortunately, the conventional culture methods for detecting colistin-resistant bacteria are not practical for monitoring food safety. Therefore, development of a simple and rapid method to detect food contamination with colistin-resistant bacteria is desirable as an effective means for preventing the dissemination of resistant bacteria, particularly colistin-resistant bacteria.

Findings: We developed a simple and rapid method for detecting *Escherichia coli* harboring the *mcr-1* colistin resistance gene using a high-speed real-time polymerase chain reaction (PCR). The entire procedure, from sample processing to final results, was performed within one hour. The practical utility of this method was verified by analyzing 27 retail meat samples for the presence of colistin-resistant bacteria. The results of the developed method were in agreement with the results of culturing colistin-resistant *E. coli* from the meat samples, demonstrating its efficacy and usefulness.

Conclusions: A simple and rapid real-time PCR-based screening method was developed for detecting *E. coli* harboring *mcr-1* in food samples. The practical utility of the procedure was confirmed using retail meat samples, indicating its potential as a convenient and rapid method to detect bacterial contamination of food items, especially in developing communities.

Introduction

Colistin is recognized as one of the few antibiotics available for the treatment of intractable infections caused by multidrug-resistant gram-negative bacteria (Giamarellou and Poulakou, 2009). Current studies have shown that colistin-resistant (CR) bacteria carrying the mobile colistin resistance gene *mcr*, which confers colistin resistance to most Enterobacteriaceae members, are widely disseminated, particularly in Asia (Shen et al., 2018; Yamamoto et al., 2018). Thus, the spread of CR bacteria in communities *via* livestock food is considered a potential risk factor because colistin is widely used in animal husbandry (Rhouma et al., 2016). Moreover, CR bacteria are often found in animals and animal-food (Kawahara et al., 2019; Yamaguchi et al., 2018; Perez-Rodriguez and Mercanoglu Taban, 2019); thus, monitoring CR bacteria in animal-food is essential. However, the conventional culture method for detecting CR bacteria in food is laborious and time-consuming. Therefore, here, we report a simple and rapid detection method for *Escherichia coli* with *mcr-1*, a CR bacterium, using a high-speed real-time polymerase chain reaction (PCR) kit. We further verified its utility for detecting CR bacteria in retail meat samples. This novel detection method holds practical relevance as the entire procedure, involving sample processing to the final result, is completed within one hour.

Materials And Methods

A total of 27 retail meat samples, such as pork and chicken, were collected from 10 markets (two supermarkets and eight local traditional markets) in Vietnam and five supermarkets in Japan during November and December 2019. In Vietnam, none of the eight traditional markets maintained a refrigerator for meat preservation. In contrast, two supermarkets in Vietnam and all supermarkets in Japan had refrigerators for food storage. Each sample was collected from one meat type per market. Bacterial cultures and DNA extraction were performed on collection day; 10 grams of each meat sample were placed in a stomacher bag (AS ONE, Osaka, Japan) containing 90 mL of buffered peptone water. The samples were hand-homogenized for 2 min. The resulting homogenate was inoculated onto CHROMagar COL-APSE (CHROMagar, France), a selective medium for CR gram-negative bacteria, and cultured at 37°C for 24 h. CR *E. coli*-like colonies were distinguished based on colony color (dark pink to reddish) after cultivation (<http://www.chromagar.com/clinical-microbiology-chromagar-col-apse-focus-on-colistin-resistance-80.html#.XkYduDL7TX4>). A representative colony was subcultured on MacConkey agar for bacterial isolation, and bacterial identification was performed. Colistin minimum inhibitory concentration (MIC) was estimated, and colistin resistance genes, *mcr-1* to *-5*, were detected by multiplex PCR as described previously (Yamaguchi et al., 2018; Wakabayashi et al., 2020).

In parallel, DNA was extracted from 1-mL homogenate (Figure 1). DNA extraction was performed using the Kaneka Easy DNA Extraction Kit version 2 (https://www.funakoshi.co.jp/exports_contents/80099, Kaneka, Tokyo, Japan).

Presence of *E. coli* and the colistin resistance gene *mcr-1* in the DNA extracts were determined by real-time PCR using a mobile PCR device, PicoGene PCR1100 (Nippon Sheet Glass, Tokyo, Japan). PCR primers and probes for real-time PCR detection of *E. coli* 16S rRNA and *mcr-1* were prepared as described previously (Table 1) (Daniels et al., 2019). Details regarding the real-time PCR, including PCR mixtures and thermal cycling conditions, are shown in Tables 2 and 3, respectively. The DNA extract of CR *E. coli* strain (E362) carrying *mcr-1* was utilized as a positive control for the PCR. The entire PCR of 50 cycles was completed in just 21 min. Moreover, this real-time PCR device simultaneously measures fluorescence at three different wavelengths for the same sample. Two fluorescent dye-labeled probes (Integrated DNA Technologies, Singapore), Cy5 for *E. coli* 16S rRNA and FAM for *mcr-1*, were used for each sample. Figure 2 shows representative real-time PCR profiles of the samples.

Table 1. Primers* and probes* used in the study

Amplified gene	Primer sequences (5'-3')
16S rRNA <i>E. coli</i> forward	TGGAGCATGTGGTTTAATTTCGA
16S rRNA <i>E. coli</i> reverse	TGCGGGACTTAACCCAACA
<i>mcr-1</i> forward	TCAGCAGTCATTATGCCAG
<i>mcr-1</i> reverse	ATACTCAATACTGGCAAGC

Probes	Sequences (5'-3')
16S rRNA <i>E. coli</i> probe	Cy5-CACGAGCTGACGACAACCATGCA-BHQ2
<i>mcr-1</i> probe	FAM-TCGCGTGCATAAGCCGCTGCGTAGCT-BHQ1

Table 2. Real-time PCR assay reaction mixture

Master mix (14 μ L)	1x KAPA Plant PCR Buffer*	8 μ L
	25 mM MgCl ₂	0.64 μ L
	20 μ M <i>mcr-1</i> primers	1.28 μ L
	20 μ M <i>E. coli</i> 16S rDNA primers	1.28 μ L
	10 μ M <i>mcr-1</i> FAM-probe	0.32 μ L
	10 μ M <i>E. coli</i> 16S rDNA Cy5-probe	0.32 μ L
	KAPA3G Plant DNA polymerase*	2.5 U/1 μ L
Template DNA extracts	2 μ L	
Total	16 μ L	

*, NIPPON Genetics, Tokyo, Japan

Table 3. Real-time PCR cycling conditions

1 step	95°C × 5 s
2 step	[95°C × 3 s → 60°C × 15 s] × 50 cycles

Results And Discussion

The detection sensitivity of the method was assessed using pork meat samples spiked with an *mcr-1*-positive *E. coli* strain culture. The lower limit of *mcr-1-E. coli* detection for the entire method, including DNA extraction to detection by real-time PCR, was 7×10^2 CFU/g, but quantification required 7×10^3 CFU/g or more due to the quantitative linearity (Fig. 3). In a validation study using retail meat samples, CR *E. coli*-like bacteria were detected using the culture method in eight out of 10 chicken and three out of seven pork samples purchased in Vietnam (Table 4).

Table 4 Detection of colistin-resistant bacteria in food

Location	Market	Presence of meat refrigerator	Sample	Culture on CHROMagar COL-APSE		Real-time PCR	
				Colistin-resistant bacteria ^a	<i>mcr-E. coli</i> ^b	Determination of <i>mcr</i>	Quantitative level ^c
Ho Chi Minh, Vietnam	H-A	Yes	Chicken	++	+	+	8.5×10^3
			Pork	-	-	-	
	H-B	No	Chicken	+++	+	+	9.8×10^5
			Pork	-	-	+	ND ^d
	H-C	No	Chicken	++	+	+	9.8×10^5
			Pork	+	+	+	4.1×10^4
	H-D	Yes	Chicken	++	+	+	ND
			Pork	-	-	-	
	H-E	No	Chicken	+++	+	+	1.1×10^8
			Pork	+	+	-	
Thai Binh, Vietnam	T-A	No	Chicken	++	+	+	1.9×10^4
			Pork	++	+	+	ND
	T-B	No	Chicken	-	-	+	1.9×10^4
			Pork	-	-	+	ND
	T-C	No	Chicken	++	+	+	1.9×10^4
T-D	No	Chicken	++	+	+	9.1×10^4	
T-E	No	Chicken	-	-	+	9.1×10^4	
Gifu, Japan	G-A,B,C,D,E	Yes	Chicken (5) ^e	-	-	-	
			Pork (5) ^e	-	-	-	

^aSemi-quantitative level of colistin-resistant bacteria colonies grown on CHROMagar COL-APSE ranging from “-” to “+++.” -, no colonies; +, dozens of colonies; ++, hundreds of colonies; +++, above the countable range.

^bPresence of *mcr-1-E. coli* identified by the culture-based method and real-time PCR.

^cNumber of bacteria harboring *mcr-1* per gram of sample.

^dND, Not determined: levels below the real-time PCR linear range.

^eTotal number of samples tested.

The semi-quantitative levels of CR bacteria in these samples were about 10^3 – 10^8 CFU/g (Table 4). All representative CR *E. coli* isolates from each sample were resistant to colistin (MIC: ≥ 4 μ g/mL) and possessed *mcr-1* but not *mcr-2* to *-5*, except the H-E pork sample which had *mcr-3* in addition to *mcr-1*, as determined by multiplex PCR. No samples from Japanese supermarkets were contaminated with CR bacteria.

All samples, except the H-E pork sample, that were positive *via* the culture-based method, were also positive by real-time PCR (Table 4). Some culture-negative samples, such as H-B pork, T-B chicken, T-B pork, and T-E chicken, were PCR-positive. Such contradictory results may be attributed to the features of the real-time PCR method and its ability to detect *mcr-1* even in dead cells. In contrast, a pork sample from the H-E market showed CR *E. coli* colonies after culturing, but tested negative for *mcr-1* by real-time PCR. The level of *mcr-1* positive bacteria may be below the detection limit of the real-time PCR method.

The new method presented here detects the target gene and facilitates quantitative analysis. The results show the ratio of bacteria carrying *mcr-1* to the number of *E. coli* cells, which may be both *mcr-1*-positive or -negative bacteria (Fig. 2). The detected quantitative *mcr-1* levels were higher than CR *E. coli*-like bacterial levels determined *via* the culture-based method because it detected all *mcr-1* regardless of bacterial species. The quantitative linear range detected *via* real-time PCR was between 10^3 and 10^6 CFU/g (Fig. 3). However, in some samples, the detected signal was below the quantitative linear range limit, yet they were considered to have positive results *via* real-time PCR.

Although the approach described in this study provided limited information regarding the degree of contamination, the developed method is reliable and practical for the rapid screening of contaminating CR bacteria in food.

Conclusions

In this study, a new, simple, and rapid screening method was developed for detecting CR *E. coli* in food samples. The developed method is advantageous because it is easy to perform, has a short processing time, and provides reliable results that are consistent with those obtained by traditional methods.

List Of Abbreviations

PCR, polymerase chain reaction; CR, colistin-resistant; MIC, minimum inhibitory concentration; CFU, colony-forming unit.

Declarations

Availability of data and materials

The datasets used and/or analyzed in this study are available from the corresponding author on reasonable request.

Competing interests

This study was conducted using a mobile PCR device, PicoGene PCR1100, provided by Nippon Sheet Glass, Tokyo, Japan. There were no competing financial interests or personal relationships that could have influenced the work reported in this paper.

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Author's contributions

HTMV participated in the study design, sample collection, real-time PCR assay and drafted the manuscript. CA-K performed the real-time PCR assay. KT participated in the study design. RK performed PCR assays and microbial analysis. DTK, TNN, HTT, CDV, and PDN contributed to sample collection and bacterial cultures. YY contributed to the study design, supervised data collection, and drafted the manuscript. All authors read and approved the final manuscript.

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Not applicable.

Authors' information

Not applicable.

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Figures

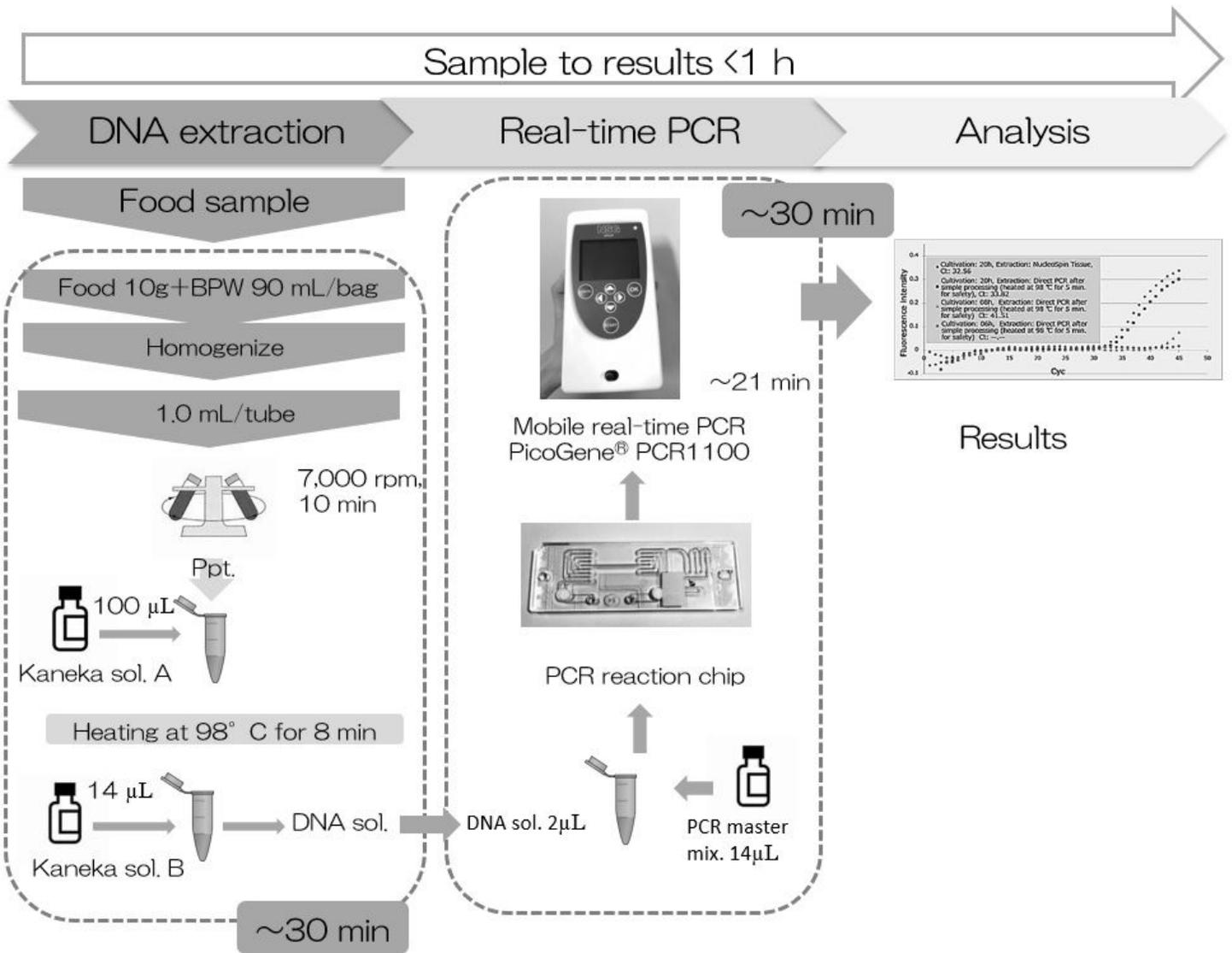


Figure 1

Outline of the screening protocol using mobile real-time PCR PicoGene® PCR1100. BPW, buffered peptone water.

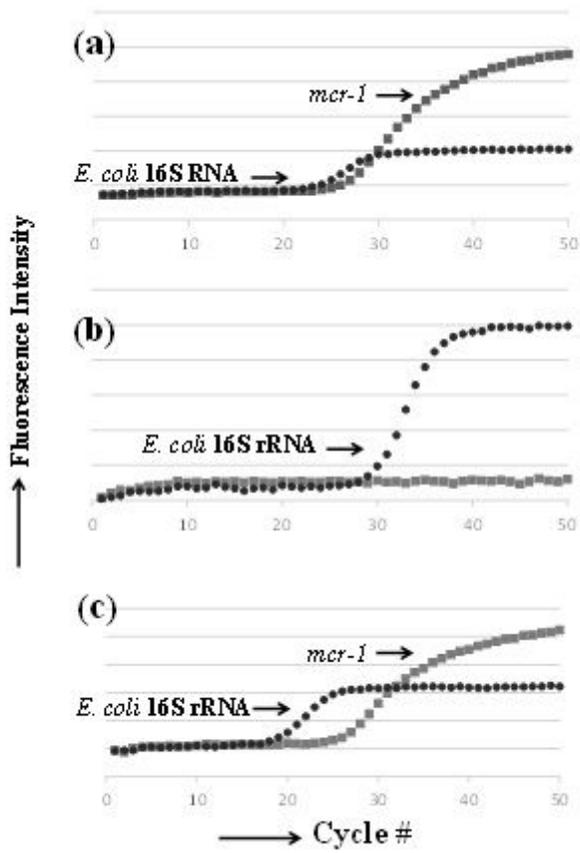


Figure 2

Representative plots obtained from real-time PCR amplification of *mcr-1* and *E. coli* 16S rRNA genes in meat samples. (a) Positive control, *mcr-1* *E. coli*. (b) *mcr-1* negative pork sample, H-A pork. (c) *mcr-1* positive chicken sample, H-E chicken.

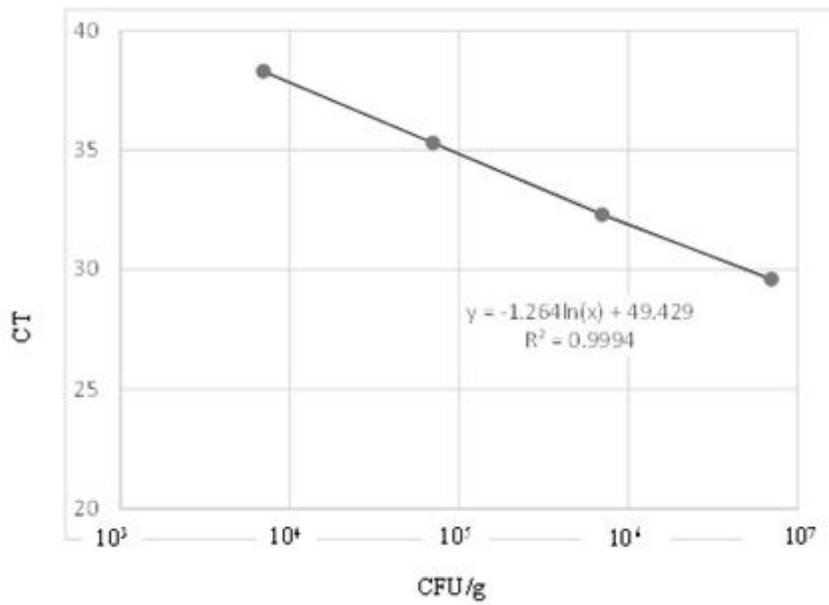


Figure 3

Standard curve of mcr-1 real-time PCR using DNA extracts from a pork meat sample spiked with mcr-1 E. coli culture. The control meat sample was spiked with mcr-E. coli (8 x 10⁶ CFU/g) and DNA was extracted as described in the text. Mean Ct values were obtained for each diluted DNA extract of three experiments and plotted. CT, cycle threshold. CFU, colony-forming unit.