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Rapid and Practical Screening Method for the Detection of Colistin-Resistant Bacteria in Food

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ABSTRACT

We developed a rapid and practical screening method for simultaneously detecting both *Escherichia coli* and bacteria harboring the *mcr-1* colistin resistance gene using high-speed real-time polymerase chain reaction with specific TaqMan probes. The entire procedure, from sample processing to the final result, was performed within 1 h. The practical utility of this method was verified by analyzing 27 retail meat samples for the presence of colistin-resistant bacteria. The results indicated the potential of this method for the convenient and rapid screening of food items to detect contamination with *mcr-1*–positive bacteria, which can be especially useful for on-site testing in developing countries.

Keywords: Colistin-Resistant Bacteria; Food; *Mcr-1*; Rapid Screening Method; Real-Time Polymerase Chain Reaction

Introduction

Colistin is recognized as one of the few remaining available antibiotics for the treatment of intractable infections caused by multidrug-resistant Gram-negative bacteria [1]. Recent studies have shown that bacteria carrying the *mcr* gene, which confers colistin resistance to most members of the Enterobacteriaceae, are widely disseminated, particularly in Asia [2,3]. Since colistin is widely used in animal husbandry [4], the spread of colistin-resistant (CR) bacteria in communities via livestock food is a potential risk factor. Moreover, CR bacteria are often found in animals and animal-food [5-7]; thus, monitoring CR bacteria in animal-food is essential. However, the conventional culture method [8] for detecting CR bacteria in food is laborious and time-consuming. Rapid detection of colistin resistance genes at the research level is now possible using the SYBR green method [9], but its widespread practicality is limited due to the need for complex steps and equipment involved in DNA extraction from samples and determination of result specificity. To overcome this limitation, we here report a simple, rapid, and practical detection method of *Escherichia coli* harboring *mcr-1*, as a representative CR bacterium, using a high-speed real-time polymerase chain reaction (PCR) kit. We further verified the utility of this method for detecting CR bacteria in retail meat samples. Although a real-time PCR assay for the detection

of *mcr* genes from bacterial isolates has already been established, this newly proposed detection method holds practical relevance for widespread use, as the entire procedure, from food sample processing to the final result, can be completed within only 1 h.

Materials and Methods

A total of 27 retail meat samples, including 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. None of the eight traditional markets in Vietnam maintained a refrigerator for meat preservation. In contrast, the two supermarkets in Vietnam and all five 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 the collection day. Ten grams of each meat sample were placed in a stomacher bag (AS ONE, Osaka, Japan) containing 90 mL buffered peptone water. The samples were hand-homogenized for 2 min. The resulting homogenate was inoculated on CHROMagar COL-APSE (CHROMagar, Paris, 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 [8,10]. A representative colony was isolated by sub-culturing on MacConkey agar, and bacterial identification was performed. The colistin minimum inhibitory concentration (MIC) was estimated, and colistin resistance genes (*mcr-1* to -5) were detected by multiplex PCR as described previously [6,11].

In parallel, DNA was extracted from 1 mL of the homogenate using the Kaneka Easy DNA Extraction Kit version 2 (Kaneka, Tokyo, Japan). The presence of *E. coli* and the colistin resistance gene *mcr-1* in the DNA extracts was determined by real-time PCR using a mobile PCR device, PicoGene PCR1100 (Nippon Sheet Glass, Tokyo, Japan). PCR primers and TaqMan probes for real-time PCR detection of *E. coli* 16S rRNA and *mcr-1* were prepared as described previously (Table 1) [12]. Details regarding the real-time PCR, including PCR mixtures and thermal cycling conditions, are provided in Tables 2 & 3, respectively. The DNA extract of the CR *E. coli* strain (E362) [6] carrying *mcr-1* was utilized as a positive control in PCR. The entire 50 PCR cycles were completed within only 21 min. Moreover, the real-time PCR device could simultaneously measure fluorescence at three different wavelengths for the same sample. Two fluorescent dye-labeled TaqMan probes (Integrated DNA Technologies, Singapore), Cy5 for *E. coli* 16S rRNA and FAM for *mcr-1*, were used for each sample. The entire protocol is outlined in Figure 1. Figure 2 shows representative real-time PCR profiles of the samples.

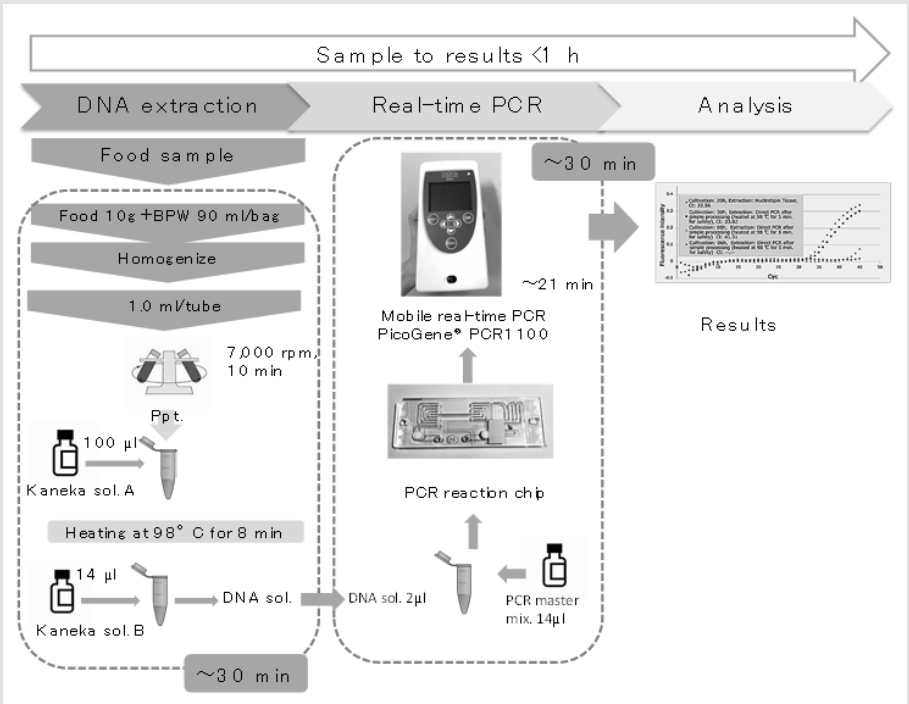

Figure 1: Outline of the screening protocol using mobile real-time PCR PicoGene® PCR1100. BPW, buffered peptone water.

Table 1: Primers and probes used in the study [12].

Amplified gene	Primer sequences (5'-3')
16S rRNA *E. coli* forward	TGGAGCATGTGGTTTAATTCGA
16S rRNA *E. coli* reverse	TGCGGGACTTAACCCAACA
mcr-1 forward	TCAGCAGTCATTATGCCAG
mcr-1 reverse	ATACTCAATACTGGCAAGC
Probes	**Sequences (5'-3')**
16S rRNA *E. coli* probe	Cy5-CACGAGCTGACGACAACCATGCA-BHQ2
mcr-1 probe	FAM-TCGCGTGCATAAGCCGCTGCGTAGCT-BHQ1

Table 2: Real-time PCR assay reaction mixture.

Master mix (14 µL)	1x KAPA Plant PCR Buffer*	8 µL
	25 mM MgCl2	0.64 µL
	20 µM *mcr-1* primers	1.28 µL
	20 µM *E. coli* 16S rDNA primers	1.28 µL
	10 µM *mcr-1* FAM-probe	0.32 µL
	10 µM *E. coli* 16S rRNA Cy5-probe	0.32 µL
	KAPA3G Plant DNA polymerase*	2.5 U/1 µL
Template DNA extracts		2 µL
Total		16 µL

Note: *NIPPON Genetics, Tokyo, Japan

Table 3: Real-time PCR cycling conditions.

Step 1	95 °C × 5 s
Step 2	[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, from DNA extraction to detection by real-time PCR, was 7 × 10^2 CFU/g; however, a minimum of 7 × 10^3 CFU/g was required for quantification using a linear correlation. In the validation study using retail meat samples, CR *E. coli*-like bacteria were detected using the culture-based method in eight out of ten chicken and in three out of seven pork samples purchased in Vietnam (Table 4). The semi-quantitative levels of CR bacteria in these samples were in the range 10^3–10^8 CFU/g (Table 4). All representative CR *E. coli* isolates from each sample were confirmed to be resistant to colistin (MIC ≥ 4 µg/mL) and possessed *mcr-1* but not *mcr*-2 to -5, except for the H-E market pork sample, which harbored *mcr*-3 in addition to *mcr-1*, as determined by multiplex PCR. No samples from the Japanese supermarkets were contaminated with CR bacteria. All samples, except for the H-E market 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 market pork, T-B market chicken, T-B market pork, and T-E market 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 and/or non-*E. coli* 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. Such discrepant cases could be due to a low level of *mcr-1*–positive bacteria below the detection limit of the real-time PCR method or the presence of bacteria expressing non-*mcr* CR determinants [13].

Table 4: Detection of colistin-resistant bacteria in food.

Location	Market	Presence of meat refrigerator	Sample	Detection on CHROMagar COL-APSE		*mcr-1*-real-time PCR	
				Colistin-resistant bacteria detection[a]	*mcr-Escherichia coli*[b]	Detection of *mcr-1*	Quantitative level[c]
Ho Chi Minh, Vietnam	H-A	Yes	Chicken	++	+	+	8.5×10^3
			Pork	-	-	-	-[d]
	H-B	No	Chicken	+++	+	+	9.8×10^5
			Pork	-	-	+	ND[e]
	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 × 104
	T-E	No	Chicken	-	-	+	9.1 × 104
Gifu, Japan	G-A,B,C,D,E	Yes	Chicken (5)[f]	-	-	-	-
			Pork (5)[f]	-	-	-	-

Note:

a. Semi-quantitative level of colistin-resistant bacteria colonies grown on a CHROMagar COL-APSE agar plate ranging from – to +++. -, no colonies; +, dozens of colonies; ++, hundreds of colonies; +++, above the countable range.

b. Presence of mcr-E. coli identified by the culture-based method and multiplex PCR.

c. Number of bacteria harboring mcr-1 (E32 positive control strain equivalent) per gram of sample.

d. Negative in real-time PCR.

e. ND: not determined; levels below the real-time PCR linear range.

f. Total number of samples tested.

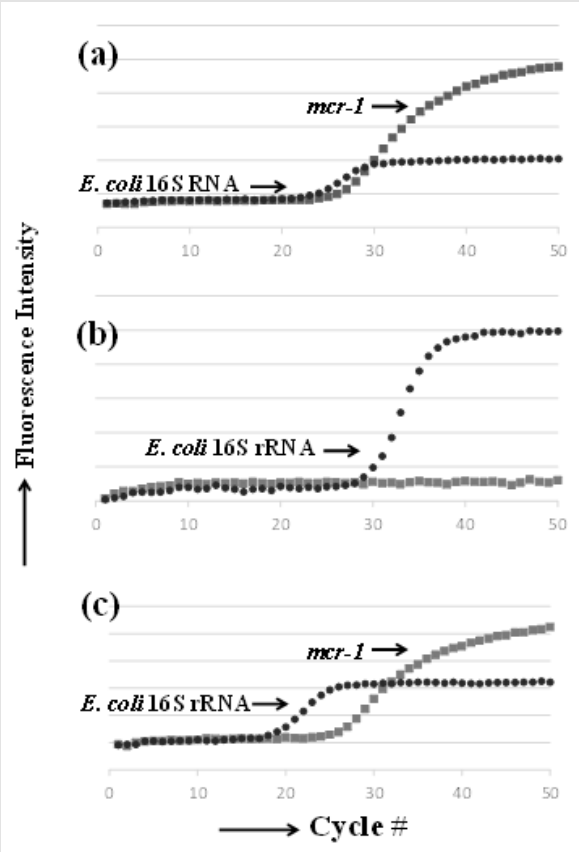

Figure 2: Representative plots obtained from real-time PCR amplification of mcr-1 and E. coli 16S rRNA genes in meat samples.
a) Positive control, *mcr-1 E. coli*.
b) *mcr-1*–negative pork sample, H-A market pork.
c) *mcr-1*–positive chicken sample, H-E market chicken.

The new method presented herein detects the target gene and facilitates quantitative analysis. In addition, the method using TaqMan probes has high detection specificity, and is simple because it does not require specificity verification by melting curve analysis, even for one-step extracted DNA from food. The results output the ratio of bacteria carrying *mcr-1* to the total number of *E. coli* cells, which may be *mcr-1*–positive or –negative bacteria (Figure 2). The detected quantitative *mcr-1* levels were higher than the CR *E. coli*-like bacterial levels determined via the culture-based method because the real-time PCR method detects 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. Although the detected signal was below the quantitative linear range limit in some samples, they were still considered to have positive results via real-time PCR. The approach described in this study provides limited information regarding the degree of contamination; nevertheless, the developed method is reliable and practical owing to a short processing time, enabling the rapid screening of contaminating bacteria with *mcr-1* in food.

Conclusion

A new rapid and practical 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.

Declarations

Ethics Approval and Informed Consent

Not applicable.

Consent for Publication

Not applicable.

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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Authors' Contributions

HV participated in the study design, sample collection, and 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.

Acknowledgment

Not applicable.

Availability of Data and Materials

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

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