A New High-Speed Droplet-Real-Time Polymerase Chain Reaction Method Can Detect Bovine Respiratory Syncytial Virus in Less than 10 Min

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ABSTRACT. The polymerase chain reaction (PCR) has been widely used for diagnosis of infectious diseases of domestic animals. Rapid detection of respiratory pathogens of cattle is useful for making therapeutic decisions. Therefore, we developed a new genetic-based method called droplet-real-time PCR, which can detect bovine respiratory syncytial virus (BRSV) within 10 min. Our droplet-real-time PCR markedly reduced the reaction time of reverse transcription-PCR while maintaining the same sensitivity as conventional real-time PCR, and it can be used as a rapid assay for detection of BRSV. Furthermore, our method is potentially applicable for rapid diagnosis of almost all infectious diseases, including highly pathogenic avian influenza virus.
KEY WORDS: bovine respiratory syncytial virus, domestic animal, droplet-real-time PCR, infectious disease.

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Rapid and accurate diagnosis of infectious diseases of domestic animals is especially critical for preventing disease spread. Although nucleic acid amplification diagnostic tests using the real-time PCR [6] are widely used to check infectious diseases [13], real-time PCR takes a considerable amount of time to obtain the results (at least several hours). A more rapid method is desirable for treatment and control of the infectious diseases. Therefore, we have developed a novel real-time PCR method that can be completed in a reaction tube within 10 min. This method utilizes two heating blocks at different temperatures—a high-temperature block (denaturation) and a low-temperature block (annealing/extension)—that can quickly alter the temperature of the reaction mixture in a newly developed PCR machine. We call this novel high-speed PCR method "droplet-real-time PCR."

Here, we applied droplet-real-time PCR to detect BRSV, which is an RNA virus classified as a pneumovirus belonging to the paramyxovirus family [12]. BRSV is indigenous in the cattle population and is common around the world. Bovine respiratory disease complex (BRD), which is frequently caused by BRSV infection of the lower respiratory tract, is a major problem in the cattle industry causing huge economic losses. Therefore, research on BRD has been a longstanding global priority. BRD accompanied with irreversible lung damage by BRSV will make livestock more susceptible to other bovine diseases and will reduce economic returns for

farmers [2, 4, 5]. For diagnosis of BRSV, virus isolation, immunohistochemistry/immunofluorescence antibody testing or reverse transcription (RT)-PCR procedures are necessary [1, 14], and these tests are both expensive and time consuming. The sensitivity of immunohistochemistry/immunofluorescence antibody testing is not high, and antibodies specific for viral protein are required. On the other hand, RT-PCR methods require a longer operation time and expensive reagents. The present study was performed to develop a rapid and cost-effective real-time RT-PCR method for detecting pathogens in animal samples.

Figure 1A shows a droplet-real-time PCR device with reaction tubes held in the holders, consisting of main and driving units. Figure 1B shows a cross-sectional view of the main unit along the line A'-A'' in Fig. 1A. The main unit is composed of 8 holders and the first and second heating blocks. The first and second heating blocks are able to heat predetermined portions of a reaction tube. The driving unit rotates the whole holder and changes the sample in the tube from the first heating block to the second or from the second to the first. The drive shaft of this device is perpendicular to the longitudinal direction of the holder, and the reaction tube in the holder is turned upside down around the drive shaft as the rotational axis. The reaction tube is filled with silicon oil. A volume of 1 μl of reaction mixture is introduced into the reaction tube with a micropipette (Fig. 1C) and is held inside the silicon oil as a spherical droplet in an internal diameter of 2 mm and a length of 25 mm. The silicon oil has a specific gravity, and the reaction mixture moves to the lowest part of the tube by gravitational force (Fig. 1C).

Droplet-real-time PCR also includes a fluorescence detector (Fig. 1A) and can also be used for conventional real-time PCR. The single fluorescence detector moves along the slide to detect appropriate fluorescence through the measurement windows of the second heating block on the lower tempera-

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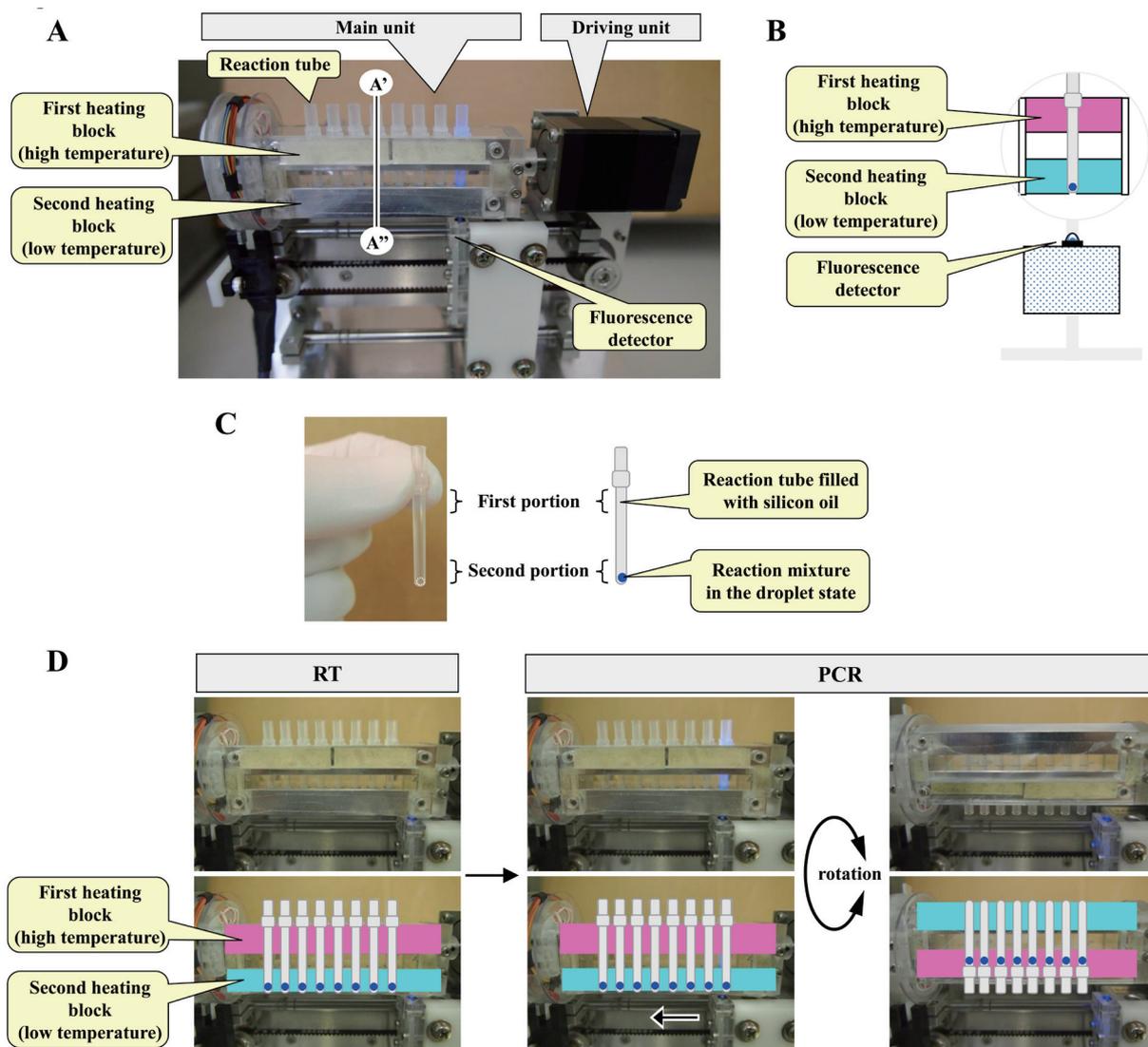
Fig. 1. Diagram of the droplet-real-time PCR device. (A) Lateral view and (B) schema of vertical cross-sectional view. (C) A photograph and a schematic image of the reaction tube. (D) Photographs and schemas during droplet-real-time RT-PCR.

Table 1. Reaction conditions of the new droplet-real-time PCR and conventional real-time PCR

The new droplet-real-time PCR				Conventional real-time PCR			
Step	Temperature	Time	Cycles	Step	Temperature	Time	Cycles
cDNA synthesis	50°C	30 s	1	cDNA synthesis	50°C	15 min	1
Inactivation of SuperScript III	98°C	10 s	1	Inactivation of SuperScript III	95°C	2 min	1
Denaturing	98°C	3 s	50	Denaturing	95°C	15 s	50
Annealing / Extension	57°C	6 s		Annealing / Extension	55°C	30 s	
Total-time	9 min 55 s			Total-time	80 min		
Machine	Droplet-real-time PCR machine			Machine	StepOnePlus Real-Time PCR		

ture side (annealing/extension step) (Fig. 1D). The first heating block heats the first portion of the reaction tube to 98°C, and the second heating block heats the second portion to 50°C (Fig. 1C and 1D). However, it improves the efficiency of droplet-real-time PCR to heat the second portion to 57°C, and we use 50 cycles of 98°C for 3 sec in the first portion and 57°C for 6 sec in the second portion. The fluorescent signals are monitored after each annealing/extension step.

Conventional real-time PCR by SrepOnePlus (Life Technologies)

Newly droplet-real-time PCR by droplet-real-time PCR machin (SEIKO EPSON)

A

B

$y = -3.1795x + 44.922$
$R^2 = 0.9994$

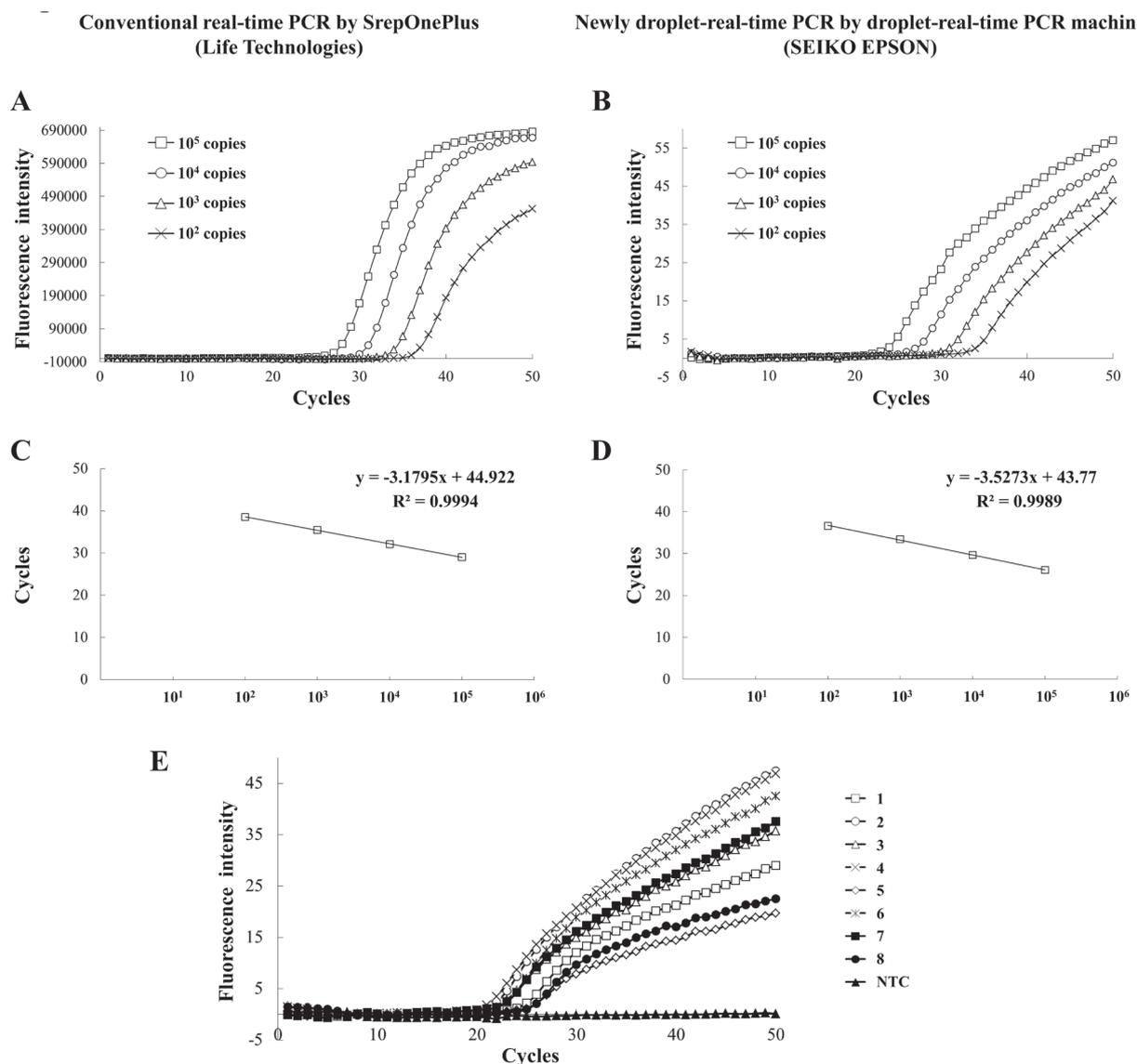**C**

$y = -3.5273x + 43.77$
$R^2 = 0.9989$

D

E

Fig. 2. (A–D) Reactivities of conventional real-time PCR and droplet-real-time PCR. Amplification plots (A and B) were obtained using 10-fold dilutions of plasmids ($10^2 - 10^5$ copies) including 138 base pairs of the BRSV nucleocapsid protein (N) gene. Standard curves were constructed from the amplification plots (C and D). (E) Reactivities of 8 BRSV-positive samples by droplet-real-time PCR. All 8 samples were positive for BRSV by droplet-real-time PCR, and the samples without BRSV were negative.

This droplet-real-time PCR can shorten the cycle time due to the simplicity of the device, and real-time PCR test can be performed within 10 min.

To compare the reactivity between droplet-real-time PCR and the conventional procedure, we examined the amplification plots using the BRSV primers and probe.

Total RNA was extracted from nasal swab samples (provided by the Nagano Prefectural Matsumoto Livestock Hygiene Service Center) suspended in diluents using a QIAamp viral RNA mini kit (Qiagen, Valencia, CA, U.S.A.) according to the manufacturer's protocol. The primers and TaqMan probe, which was used in real-time RT-PCR to detect BRSV, were 5′-GGTCAAACTAAATGACACTTTCAACAAG-3′

(forward) and 5′-AGCATACCACACAACTTATTGAGA-TG-3′ (reverse), 5′-FAM-TAGTACAGGTGACAACATTG-BHQ-3′ [9]. The droplet-real-time RT-PCR mixture contained RNA, SuperScript III/Platinum Taq Mix (Life Technologies, Grand Island, NY, U.S.A.), 5× reaction buffer, 1.6 μmol/l of each primer and 0.4 μmol/l of TaqMan probe in a total volume of 10 μl. Aliquots of 1 μl of the reaction mixture were used for droplet-real-time PCR (Seiko Epson, Nagano, Japan), and 25 μl of the same mixture was used for conventional real-time PCR (Life Technologies, Carlsbad, CA, U.S.A.). Reaction conditions are described in Table 1. Amplification plots were obtained using 10-fold dilutions of plasmids ($10^2 - 10^5$ copies) carrying the 138 base pairs

of the BRSV nucleocapsid protein (N) gene (TaKaRa Bio, Yokkaichi, Japan). All experimental procedures were carried out in accordance with the regulations of animal experimentation of Shinshu University.

The reaction time of droplet-real-time PCR was 10 min, whereas that of conventional real-time PCR was 80 min. The standard curve constructed from the amplification plots by droplet-real-time PCR using 10-fold dilutions of plasmids including a portion of the BRSV nucleocapsid protein (N) gene ($10^2 - 10^5$ copies) was: y= −3.5273x + 43.77; that by conventional real-time PCR was: y= −3.1795x + 44.922 (Fig. 2A, 2B, 2C and 2D). Droplet-real-time PCR was as sensitive as conventional real-time PCR. Following droplet-real-time PCR, droplets were collected from the reaction tube and analyzed by gel electrophoresis. The amplified products were of the expected length, and no byproducts were observed (data not shown). All eight clinical samples (provided by the Nagano Prefectural Matsumoto Livestock Hygiene Service Center), which were positive for BRSV by conventional real-time PCR, were also positive using the droplet-real-time PCR (Fig. 2E).

Droplet-real-time PCR was able to detect BRSV within 10 min compared to 80 min for conventional real-time PCR (not including the time for RNA extraction). It has two heating blocks at different temperatures so that the reaction mixture can move by gravity to the incubator at the optimal temperature during rotation of the reaction tube (Fig. 1). Therefore, time to change the temperature of the reaction mixture is not required in the droplet-real-time PCR machine, but is required in the conventional real-time PCR machine with only one thermal incubator. These features of the droplet-real-time PCR machine enable high-speed reaction. The reaction conditions, which included thermal conditions, reagent concentrations and reaction buffer, were also important to shorten the reaction time. Therefore, droplet-real-time PCR markedly reduced the reaction time of RT-PCR while maintaining the same sensitivity as conventional real-time PCR (Fig. 2).

Other genetic diagnostic methods, such as the loop-mediated isothermal amplification (LAMP) [7], have recently become available. The reverse transcription (RT)-LAMP method involves simple procedures and has high sensitivity; however, this method requires 60 min for the reaction [10]. The present droplet-real-time PCR procedure was capable of detecting BRSV in a total time of less than 10 min. Thus, it can play a critical role in rapid diagnosis of serious infectious diseases in domestic animals. Droplet-real-time PCR can also be applied to detect other pathogens, such as bovine herpesvirus-1 (BoHV-1, also known as bovine infectious rhinotracheitis virus), bovine parainfluenza type 3 (BPI3) [3, 8] and highly pathogenic avian influenza viruses [11]. If genetic tests can be performed by droplet-real-time PCR on the farm without sending the samples to a laboratory, effective countermeasures can be quickly carried out in the early phase of a serious pathogen outbreak. This study demonstrated the potential application of droplet-real-time PCR to rapidly detect viral (microbial) RNA/DNA in clinical specimens. In addition, its additional advantages are smaller sample volume, cheaper reagent costs and shorter assay turnaround time compared to conventional real-time PCR. However, droplet-real-time PCR requires a DNA/RNA extraction process, and it will be necessary to develop a simple and rapid method to extract RNA/DNA to reduce the total time for diagnosis of disease-causing pathogens.

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