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Simultaneous and rapid detection method for measles and rubella using single-tube multiplex real-time quantitative RT-PCR☆

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ABSTRACT

Patients with measles or rubella infections manifest acute onset fever accompanying systemic exanthema, which are clinically difficult to be distinguish. Rapid diagnosis and differentiation of such epidemic viral diseases is essential to prevent outbreaks. We developed a single-tube multiplex real-time PCR assay for these indistinguishable viruses. We used previously-reported primer settings, with a slight modification of reporter dye, and applied to multiplex Taqman real-time PCR by cobas z480 (Roche Molecular Systems, Inc.). Consequently, the assay could detect 10 copies/10 μl of measles and rubella with coefficient of variations of 11.2% and 21.8%, respectively. Strengths of our methodology include simplicity of operation, short measurement time (2 h), uses of internal control (confirming a run of PCR), and quantitative measurement with high sensitivity. Both measles and rubella currently cause social outbreaks in Japan. We hope that our single-tube multiplex assay contributes to an early diagnosis, leading to an appropriate infection control measure and prevention of epidemics.

Measles and rubella have been targeted for global elimination by the World Health Organization (WHO) [1], which requires a well-established surveillance system to comprehend the current trends of the diseases [2]. In Japan, measles has been declared by the WHO to be eliminated by 2015 [3]. However, sporadic measles outbreaks have still occurred as a result of imported infections [4,5]. Rubella currently impacts the Japanese society as well, particularly by increasing the incidence of congenital rubella syndrome [6,7]. Clinical presentations of measles and rubella are quite similar and differentiating these viral diseases, particularly in an early stage of the clinical course, is often challenging. Development of multiplex detection for these viruses has long been underway and several methodologies have been reported in the literature to date [8–12]. To establish a simpler and faster method, we herein report another approach for simultaneous detection of measles and rubella using multiplex real-time quantitative reverse transcription-PCR (qRT-PCR) test.

A 20 μL reaction mixture was composed of 10 μL of sample templates and 10 μL reaction master mix, consisting of TaqMan Fast Virus 1-Step Master Mix (5 μL) (Thermo Fisher Scientific, Inc., Japan), 1 μL each of primer mixtures for measles and rubella (forward primer [8 μM], reverse primer [8 μM], and probe [5 μM] for measles; forward primer [18 μM], reverse primer [18 μM], and probe [5 μM] for rubella), 0.5 μL of LightMix® Modular PhHV Internal Control (Roche Diagnostics K.K), 2.5 μL of Nuclease-Free Water (Thermo Fisher Scientific Inc., Japan). The primers and probes applied to multiplex qRT-PCR are summarized in Table 1. We referred to previous publications for the primer settings of measles and rubella with a slight modification of the reporter dye [13–15]. Multiplex qRT-PCR assay was performed using cobas® z480 (Roche Molecular Systems, Inc.) under a following PCR condition: reverse transcription at 50 °C for 5 min (1 cycle), an initial denaturation at 90 °C for 20 s (1 cycle), followed by 50 cycles at 95 °C for 15 s (amplification) and 60 °C for 1 min (extension). Wavelengths examined were at 510 nm for measles, 580 nm for rubella, and 670 nm for the internal control. RNA standards of measles and rubella was adjusted to 100,000 copies/10 μL and serial-diluted 10-

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Table 1
Primers and probes used in the multiplex qRT-PCR test.

	Primer names	Sequences (5′ - 3′)
Measles	MVN1139F	TGGCATCTGAACTCGGTATCAC
	MVN1213R	TGTCCTCAGTAGTATGCATTGCAA
	MVNP1163Probe	(FAM)-CCGAGGATGCAAGGCTTGTTTCAGA-(TAMRA)
Rubella	NS(32–54)For	CCTAHYCCCATGGAGAAACTCCT
	NS(143–160)Rev	AACATCGCGCACTTCCCA
	NS(93–106)Probe	(VIC)-CCGTCGGCAGTTGG-(MGB)

Originally, a reporter dye of NS (93–106) Probe was FAM, instead of VIC. The primer settings were based on previous reports [13–15].

fold with nuclease-free water to build standard curves. For the confirmation of reproducibility of the assay, we applied vaccine strains available (Takeda Pharmaceutical Co., Ltd., Japan). A qualitative detection sensitivity was set at the lowest concentrations of the RNA standards measured.

First, we confirmed that the measles RNA was not detectable in the single rubella detection assay and vice versa (data not shown). As a result of triplicate experiments for developing standard curve (Fig. 1), averages ±2 standard deviations of the slopes for measles and rubella were −3.5 ± 0.1 and −3.3 ± 0.1, respectively. Also, R-squared values, or coefficient of determinations, were 0.993 ± 0.001 for measles and 0.995 ± 0.008 for rubella. Five times repeated experiments showed that qualitative detection sensitivities of the assay were 10 copies/10 μL for both measles and rubella. As for lower limits of quantification, the assay could detect 10 copies/10 μl of measles and rubella with coefficient of variations of 11.2% and 21.8%, respectively. We confirmed reproducibility of the multiplex qRT-PCR assay by repeating independent experiments five times each for five different viral concentrations (10^1 - 10^5) (Supplement Table 1). The total procedure time for this assay was approximately 2 h.

Several articles have been published on multiplex qRT-PCR assay targeting measles and rubella. The first such challenge was reported by Mosquera et al. in 2002 [8]. They used several clinical samples including sera, pharyngeal exudates, cerebrospinal fluid, and urine, and concluded the high sensitivity of their testing with detection limit of 10 copies for both measles and rubella. They included Parvovirus B19 in the multiplex assay as well. However, viral amplification was confirmed by gel electrophoresis, making the process laborious, complicated, potentially contaminating, and time-consuming. Subsequently, accuracy of the assay was re-evaluated using 229 pharyngeal specimens, and sensitivity of the assay for rubella was shown to be only 42.6% [9]. Then, an independent study by Hübschen et al. established the multiplex Taqman PCR assay for measles and rubella in 2008 [10]. The study has an advantage in applying multiple genotypes of the two viruses, including both clinical and reference strains, but was limited by a lack of applicability to clinical samples. In 2012, Kaida et al. developed the multiplex Taqman RT-PCR assay targeting 5 viral diseases; measles, rubella, Parvovirus B19, human herpes virus-6, and human herpes virus-7 [11]. Although 187 clinical samples were analyzed, their method was lack of the internal control and a quantitative performance. In 2013, another multiplex Taqman RT-PCR assay targeting 3 viral diseases (measles, rubella, and mumps) was introduced, which, however, did not apply an internal control in the assay [12].

Compared to these preceding assays, our assay has several notable strengths. First, all the tests are performed using an internal control, which promises correct running of the PCR. Second is simplicity of the operation; *i.e.* only a dispensing process of samples and reagents is required between full-automatic RNA extraction and PCR process. RNA is vulnerable and a process with less manipulations prevents the accidental degradation of samples. The third point that should be considered is the short period of measurement time; *i.e.* totally 2 h. Fourth, quantitative measurement of the assay with lower limits of quantification up to ≥10 copies/10 μL is a major advantage. As described here, the method we constructed can be used as a quick and convenient method for simultaneous detection of measles and rubella in clinical laboratories.

Limitations of the assay should also be mentioned. Absence of experiments using clinical samples is the major weakness. In the community, there circulates various genotypes of measles and rubella. We have not yet applied these clinical strains with genetic diversity, although the assay successfully detected commercially available measles or rubella vaccine strains. We still cannot conclude the clinical usefulness of this new assay, and further studies including clinical samples will be crucial in corroborating the accuracy and availability of our method in clinical settings.

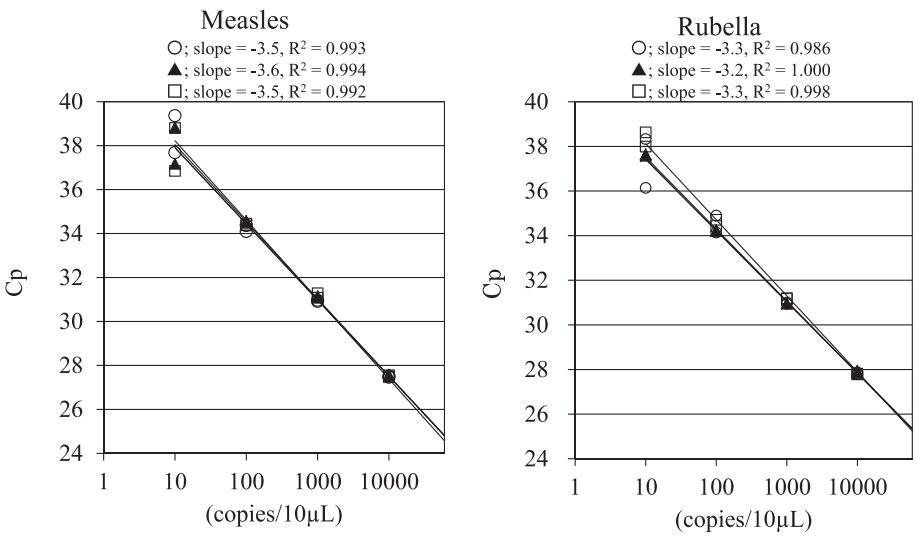

Fig. 1. Standard curve for ten-fold serial dilutions of the RNA standards. The log numbers of copies/10 μL on the x-axis; Cp values on the y-axis. A line indicates a linear detection range.

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In conclusion, we have developed a multiplex RT-PCR assay for the simultaneous detection of measles and rubella. Manifestations of these viral infections clinically resemble each other, and we postulate that our methodology paves the way for a simpler yet efficient and speedy screening test.

Conflicts of interest

The authors have no conflicts of interests to declare.

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Authors' contributions

Study conception, design, and acquisition and interpretation of data: N. Yoshioka, M. Deguchi, and M. Kagita. Drafting of the manuscript: H. Hagiya. Critical revision: S. Hamaguchi and K. Tomono.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jiac.2019.05.005.

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