Table 4
Sensivity comparisons of the primer sets: test results (P* or N**) with the time (minutes) when turbidity signals passed the cutoff.

Strain		Viral dose (PFU)							
		10^3		10^2		10^1		10^0	
	NSP***	E1*	NSP	E1	NSP	E1	NSP	E1	
Rvi/Dezhou.CHN/02	1E	P (19.25 ± 0.52)****	P (23.81 ± 1.39)	P (27.68 ± 1.62)	P (27.92 ± 2.15)	N (>60.0)	N (>60.0)	N (>60.0)	N (>60.0)
Rvi/Minsk.BLR/28.05	1H	P (18.33 ± 1.40)	P (24.17 ± 1.68)	P (24.01 ± 3.67)	P (27.82 ± 2.71)	N (>60.0)	N (>60.0)	N (>60.0)	N (>60.0)
Rvi/Miyazaki.JPN/10.01 CRS	1J	P (18.04 ± 0.64)	P (28.81 ± 2.02)	P (24.28 ± 3.97)	P (36.08 ± 5.24)	N (>60.0)	N (>60.0)	N (>60.0)	N (>60.0)
Rvi/Telaviv.ISR/68	2B	P (22.08 ± 2.25)	N (>60.0)	N (>60.0)	N (>60.0)	N (>60.0)	N (>60.0)	N (>60.0)	N (>60.0)

* P: positive.
** N: negative.
*** NSP and E1 are the primer sets used in this study and previously reported, respectively.
**** Data represent the mean values calculated from three independent experiments.

shows the average values, as the times (in minutes) when the turbidity signal was beyond the threshold. The data indicated that 100–1000 PFU/reaction of virus was required for detection by the new RT-LAMP assay, except for the genotype 2C virus (RVi/Moscow.RUS/67).

The assay failed to detect the genotype 2C virus, whereas the TaqMan real-time PCR assay could detect 10 PFU/reaction of this virus (data not shown). The geographical location of genotype 2C viruses was restricted to Russia, and genotype 2C viruses have not been detected since 2005. Thus, the problem in detecting genotype 2C viruses is likely not an important obstacle for the new RT-LAMP assay. However, this problem may require consideration when using the assay.

It is necessary to distinguish rubella from measles that has the similar clinical symptoms with rubella. The specificity of the new RT-LAMP assay was tested using measles virus RNA derived from genotype D3, D5, D8, and H1. No amplification was found with the RT-LAMP assay even with about 6 $\log_{10}$ copies/reaction, thus the RT-LAMP assay was thought not to cross-react with measles virus genomes (data not shown).

The sensitivity of the new NSP primer set was compared with the primer set reported previously by Mori et al. (2006). The primer set used by Mori et al. was targeted to the E1 structural protein gene (Table 1), and was thus designated the "E1 primer set" in this study. RNA extracts from virus stocks of four major genotypes circulating in the world were subjected to the RT-LAMP assay using either the NSP primer set or E1 primer set (Table 4). Both assays detected 100 PFU of RV genotypes 1E (Rvi/Dezhou.CHN/02), 1h (Rvi/Minsk.BLR/28.05), and 1J (Rvi/Miyazaki.JPN/10.01 CRS), suggesting that for RV genotypes 1E, 1H, and 1J, the sensitivity of the assay targeting the non-structural protein gene was comparable to that targeting the E1 gene reported by Mori et al. (2006). On the other hand, the E1 primer set failed to detect the genotype 2B RV (Rvi/Telaviv.ISR/68), while the NSP primer set detected it successfully, suggesting that the NSP primer set was useful for detection of 2B genotype viruses. Mori et al. (2006) described that the detection limit of their assay was approximately 1 PFU of virus. The difference in the detection limits between the assays may arise from differences in determination of the detection limits by PFU. Specifically, diluted viral RNA serially after extraction from virus stock was used previously while we used viral RNA extracted from serially diluted virus stocks to more closely resemble actual specimens with low amount of virus. The E1 primer set exhibited an almost equivalent detection limit to the NSP primer set under the conditions used in this study (Table 4). Finally, a spike test was performed using a similar procedure to previously reported method (Okamoto et al., 2010). Briefly, four viral strain stocks, Rvi/Dezhou.CHN/02[1E], Rvi/Minsk.BLR/28.05[1h], Rvi/Telaviv.ISR/68[2B], and Rvi/Miyazaki.JPN/10.01 CRS[1j], were diluted to 10^5, 10^4, 10^3, 10^2, and 10^1 PFU/ml with throat swabs from healthy donors collected in Universal Viral Transport Medium (BD, Franklin Lakes, NJ), and viral RNAs were extracted from 140 μl aliquots of these samples. Five microliters of each viral RNA extract was subjected to the RT-LAMP assay using the NSP primer set (Table 5). For all of the tested genotypes, the spike test showed similar sensitivity to that using viral RNA extracted from culture medium (Tables 4 and 5). Therefore, neither the RNA extraction steps nor the amplification steps of the RT-LAMP assay were affected by the presence of RNAs typically found in normal throat swabs.

In this study, the RT-LAMP assay was improved for detection of RV strains currently circulating in the world. The improved assay detected successfully RV genotypes 1E, 1H, 1J, and 2B, the current major genotypes worldwide. Therefore, the improved RT-LAMP assay should be a useful assay for laboratory diagnosis of rubella. The assay is less sensitive than some real-time assays, a fact which should be considered when dealing with samples containing small amount of RV RNA. However, the RT-LAMP assay can be used for rapid laboratory diagnosis, does not require sophisticated devices like real-time PCR systems, and decreases the risk of laboratory contamination because of the lack of handling procedures for the amplified products. Surveillance for rubella and CRS using RNA detection for laboratory confirmation is problematic in many countries; the improved assay described here could improve RNA confirmation in these countries.

Table 5
Spike tests: test results (P* or N**) with the time (minutes) when turbidity signals passed the cutoff.

Strain		Viral dose (PFU)			
	10^3	10^2	10^1	10^0	
Rvi/Dezhou.CHN/02	1E	P (22.74 ± 4.38)***	P (29.78 ± 4.01)	N (>60.0)	N (>60.0)
Rvi/Minsk.BLR/28.05	1H	P (20.12 ± 1.39)	P (26.24 ± 2.57)	N (>60.0)	N (>60.0)
Rvi/Telaviv.ISR/68	2B	P (22.30 ± 0.30)	N (>60.0)	N (>60.0)	N (>60.0)
Rvi/Miyazaki.JPN/10.01 CRS	1J	P (25.36 ± 4.90)	N (>60.0)	N (>60.0)	N (>60.0)

* P: positive.
** N: negative.
*** Data represent the mean values calculated from three independent experiments.

Disclaimer

The findings and conclusions in this report are those of the author(s) and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

Acknowledgements

This work was supported by Grants-in-Aid from the Ministry of Health, Labour and Welfare, Science Research Grant(s) (H24-Iyaku-Ippan-008 and H25-Shinko-Ippan-010). We thank Dr. P. Rota and all members of Measles, Mumps, Rubella and Herpes Virus Laboratory Branch, Division of Viral Diseases, Centers for Disease Control and Prevention, for their helpful discussions.

References

Abernathy, E.S., Hübschen, J.M., Muller, C.P., Jin, L., Brown, D., Komase, K., Mori, Y., Xu, W., Zhu, Z., Siqueira, M.M., Shulga, S., Tikhonova, N., Pattamadilok, S., Incomserb, P., Smit, S.B., Akoua-Koffi, C., Bwogi, J., Lim, W.W., Woo, G.K., Triki, H., Jee, Y., Mulders, M.N., de Filippis, A.M., Ahmed, H., Ramamurty, N., Featherstone, D., Icenogle, J.P., 2011. Status of global virologic surveillance for rubella viruses. J. Infect. Dis. 204 (Suppl. 1), S524–S532.

Bellini, B., Icenogle, J., 2011. Measles and rubella. Man. Clin. Microbiol. 2, 1378–1387.

CDC, 2008. Recommendations from an ad hoc Meeting of the WHO Measles and Rubella Laboratory Network (LabNet) on use of alternative diagnostic samples for measles and rubella surveillance. Morb. Mortal. Wkly. Rep. 57, 657–660.

Frey, T.K., 1994. Molecular biology of rubella virus. Adv. Virus Res. 44, 69–160.

Goto, M., Honda, E., Ogura, A., Nomoto, A., Hanaki, K., 2009. Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxy naphthol blue. Biotechniques 46, 167–172.

Ito, M., Watanabe, M., Nakagawa, N., Ihara, T., Okuno, Y., 2006. Rapid detection and typing of influenza A and B by loop-mediated isothermal amplification: comparison with immunochromatography and virus isolation. J. Virol. Methods 135, 272–275.

Le Roux, C.A., Kubo, T., Grobbelaar, A.A., van Vuren, P.J., Weyer, J., Nel, L.H., Swanepoel, R., Morita, K., Paweska, J.T., 2009. Development and evaluation of a real-time reverse transcription-loop-mediated isothermal amplification assay for rapid detection of Rift Valley fever virus in clinical specimens. J. Clin. Microbiol. 47, 645–651.

Liang, Y., Gillam, S., 2000. Mutational analysis of the rubella virus nonstructural polyprotein and its cleavage products in virus replication and RNA synthesis. J. Virol. 74, 5133–5141.

Mori, Y., Nagamine, K., Tomita, N., Notomi, T., 2001. Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochem. Biophys. Res. Commun. 289, 150–154.

Mori, N., Motegi, Y., Shimamura, Y., Ezaki, T., Natsumeda, T., Yonekawa, T., Ota, Y., Notomi, T., Nakayama, T., 2006. Development of a new method for diagnosis of rubella virus infection by reverse transcription-loop-mediated isothermal amplification. J. Clin. Microbiol. 44, 3268–3273.

Nagamine, K., Hase, T., Notomi, T., 2002. Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol. Cell. Probes 16, 223–229.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., Hase, T., 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 28, E63.

Okamoto, K., Fujii, K., Komase, K., 2010. Development of a novel TaqMan real-time PCR assay for detecting rubella virus RNA. J. Virol. Methods 168, 267–271.

Oker-Blom, C., Ulmanen, I., Kääriäinen, L., Pettersson, R.F., 1984. Rubella virus 40S genome RNA specifies a 24S subgenomic mRNA that codes for a precursor to structural proteins. J. Virol. 49, 403–408.

Pugachev, K., Abernathy, E., Frey, T., 1997. Improvement of the specific infectivity of the rubella virus (RUB) infectious clone: determinants of cytopathogenicity induced by RUB map to the nonstructural proteins. J. Virol. 71, 562–568.

Reddy, V., Ravi, V., Desai, A., Parida, M., Powers, A.M., Johnson, B.W., 2012. Utility of IgM ELISA, TaqMan real-time PCR, reverse transcription PCR, and RT-LAMP assay for the diagnosis of Chikungunya fever. J. Med. Virol. 84, 1771–1778.

Reed, L.J., Muench, H., 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. 27, 493–497.

Ushio, M., Yui, I., Yoshida, N., Fujino, M., Yonekawa, T., Ota, Y., Notomi, T., Nakayama, T., 2005. Detection of respiratory syncytial virus genome by subgroups-A, B specific reverse transcription loop-mediated isothermal amplification (RT-LAMP). J. Med. Virol. 77, 121–127.

WHO, 2008. Measles and rubella laboratory network: 2007 meeting on use of alternative sampling techniques for surveillance. Wkly. Epidemiol. Rec. 83, 225–232.

WHO, 2011. Rubella vaccines: WHO position paper. Wkly. Epidemiol. Rec. 86, 301–316.

WHO, 2013. Rubella virus nomenclature update. Wkly. Epidemiol. Rec. 88 (32), 337–343.

Yao, J., Yang, D., Chong, P., Hwang, D., Liang, Y., Gillam, S., 1998. Proteolytic processing of rubella virus nonstructural proteins. Virology 246, 74–82.

Zhu, Z., Xu, W., Abernathy, E.S., Chen, M., Zheng, Q., Wang, T., Zhang, Z., Li, C., Wang, C., He, W., Zhou, S., Icenogle, J., 2007. Comparison of four methods for confirming rubella virus infection using throat swabs. J. Clin. Microbiol., 452847–452852.

Shirato et al. Virology Journal 2014, 11:139
http://www.virologyj.com/content/11/1/139

VIROLOGY JOURNAL

RESEARCH Open Access

Detection of Middle East respiratory syndrome coronavirus using reverse transcription loop-mediated isothermal amplification (RT-LAMP)

Kazuya Shirato[1*], Takuya Yano[2], Syouhei Senba[3], Shigehiro Akachi[2], Takashi Kobayashi[2], Takamichi Nishinaka[2], Tsugunori Notomi[3] and Shutoku Matsuyama[1]

Abstract

Background: The first documented case of Middle East Respiratory Syndrome coronavirus (MERS-CoV) occurred in 2012, and outbreaks have continued ever since, mainly in Saudi Arabia. MERS-CoV is primarily diagnosed using a real-time RT-PCR assay, with at least two different genomic targets required for a positive diagnosis according to the case definition of The World Health Organization (WHO) as of 3 July 2013. Therefore, it is urgently necessary to develop as many specific genetic diagnostic methods as possible to allow stable diagnosis of MERS-CoV infections.

Methods: Reverse transcription-loop-mediated isothermal amplification (RT-LAMP) is a genetic diagnostic method used widely for the detection of viral pathogens, which requires only a single temperature for amplification, and can be completed in less than 1 h. This study developed a novel RT-LAMP assay for detecting MERS-CoV using primer sets targeting a conserved nucleocapsid protein region.

Results: The RT-LAMP assay was capable of detecting as few as 3.4 copies of MERS-CoV RNA, and was highly specific, with no cross-reaction to other respiratory viruses. Pilot experiments to detect MERS-CoV from medium containing pharyngeal swabs inoculated with pre-titrated viruses were also performed. The RT-LAMP assay exhibited sensitivity similar to that of MERS-CoV real-time RT-PCR.

Conclusions: These results suggest that the RT-LAMP assay described here is a useful tool for the diagnosis and epidemiologic surveillance of human MERS-CoV infections.

Keywords: Meddle East respiratory syndrome (MERS), MERS coronavirus (MERS-CoV), RT-LAMP, Genetic diagnostic method

Background

On 22 September 2012, a novel coronavirus sequence was detected from a 49-year-old patient presenting with severe pneumonia who was initially treated in an intensive care unit in Qatar and then moved to London [1]. The sequence of the PCR amplicon of this isolate was a close match with that of a coronavirus isolated from a 60-year-old patient who had died of severe pneumonia in Jeddah, Saudi Arabia in June 2012 [1,2]. Together, these two cases marked the beginning of an outbreak of severe respiratory infections caused by a newly identified coronavirus, designated the Middle East Respiratory Syndrome coronavirus (MERS-CoV) [3]. This outbreak is ongoing, with 836 confirmed cases to date that have resulted in 288 deaths in 19 countries (Jordan, Qatar, Saudi Arabia, the United Arab Emirates, Oman, Kuwait, Yemen, Lebanon, Iran, Algeria, Tunisia, France, the Netherlands, Germany, the United Kingdom, Greece, Malaysia, Philippines and the United States of America) as of 14 July, 2014 [The World Health Organization (WHO), Global Alert and Response (GAR), Coronavirus infections, updated on 14 July 2014, http://www.who.int/csr/disease/coronavirus_infections/en/index.html].

Sequence analyses show that MERS-CoV clusters with the group 2c betacoronavirus, and is closely related to

* Correspondence: shirato@nih.go.jp
[1]Laboratory of Acute Respiratory Viral Diseases and Cytokines, Department of Virology III, National Institute of Infectious Disease, Laboratory of Acute Respiratory Viral Diseases and Cytokines, 4-7-1 Gakuen, Musashimurayama, Tokyo 208-0011, Japan
Full list of author information is available at the end of the article

the bat coronaviruses HKU4 and HKU5 [4]. Severe acute respiratory syndrome coronavirus (SARS-CoV), which caused severe pneumonia resulting in 8,098 reported infections and 774 deaths between 2002 and 2003 [5], was also derived from bat coronaviruses [6,7]. MERS-CoV, with its similar symptoms and phylogeny, is therefore considered a cousin of SARS-CoV. The reservoir for MERS-CoV remains unclear, but recent reports suggest that camels are the most likely candidate, as a form of the virus has been circulating in camels in Saudi Arabia since at least 1992 [8-13].

MERS-CoV is primarily diagnosed using a real-time RT-PCR assay, and at least two different genomic targets are required for a positive diagnosis. The first probe and primer sets for MERS-CoV detection by real-time PT-PCR were developed by Corman *et al.* shortly following the first reports of the disease [14,15]. Among them, the probe and primer sets targeting upE and ORF1a exhibit the highest sensitivities, and remain the most widely used targets for MERS-CoV detection. At least two different specific genomic targets are required for a positive diagnosis according to the case definition announced by the WHO as of 3 July 2013 [WHO, GAR, Revised interim case definition for reporting to WHO – Middle East respiratory syndrome coronavirus (MERS-CoV), updated on 3 July 2013, http://www.who.int/csr/disease/coronavirus_infections/case_definition/en/index.html]. A single positive target followed by gene sequencing is also considered positive; however, the current gene sequencing technique requires PCR amplicons, and the ability of conventional RT-PCR to produce a sequencing-quality template is generally lower than that of real-time RT-PCR [16-20]. Therefore, it is urgently necessary to develop as many specific genetic diagnostic methods as possible to allow reliable diagnosis of MERS-CoV infections.

The loop-mediated isothermal amplification (LAMP) method amplifies specific nucleic acid sequences using a set of four or six unique primers [21,22]. The LAMP procedure is user-friendly, since the reaction mixture is incubated at a single temperature for less than 1 h. Amplification can be detected as the precipitation of magnesium pyrophosphate or by fluorescence under ultra-violet light, and also can be detected in real time by monitoring the turbidity of the pyrophosphate [23]. The LAMP assay can also be used for the detection of RNA by combining reverse transcription with LAMP (RT-LAMP) [21]; RT-LAMP assays have been developed for a variety of respiratory RNA viruses, including SARS [24], respiratory syncytial virus [25,26], and influenza viruses [27,28]. In this study, a novel RT-LAMP method for the detection of MERS-CoV was developed, with a sensitivity similar to that of real-time RT-PCR targeting upE and ORF1a.

Materials and methods

Viruses

The MERS-CoV EMC isolate was kindly provided by Ron A. M. Fouchier, Erasmus Medical Center, Rotterdam, The Netherlands. MERS-CoV was propagated and titrated using Vero cells. Human respiratory syncytial viruses (RSV; Long, A2, B WV/14617/85 and 18537) were obtained from the American Tissue Culture Collection (ATCC). Human metapneumovirus (HMPV; Sendai-H/2404/2003) was obtained from the Virus Research Center, Sendai Medical Center, Japan. Human coronavirus (HCoV)-229E isolates ATCC VR-740 and Sendai-H/1121/04 [29] were used. HCoV-NL63 was supplied by Dr. Hoek, University of Amsterdam, Netherlands. Isolate HCoV-OC43 was obtained from ATCC. SARS coronavirus (Frankfurt strain) was supplied by Dr. J. Ziebuhr, University of Würzburg, Germany. Human parainfluenza viruses (PIV) 1 (strain C35) and 3 (strain C243) were obtained from ATCC. Adenoviruses (ADV) (serotype 3, strain G.B.; serotype 4, strain RI-67; and serotype 7, strain Gomen) were obtained from ATCC. Viruses were propagated and titrated using HEp-2, HeLa, RD, Vero cells, or LLC-Mk2 cells [30]. Influenza viruses [Flu; A/California/7/2009 (H1N1pdm), A/Victoria/210/2009 (H3N2), and B/Brisbane/60/2008] were provided by the Influenza Virus Research Center of the National Institute of Infectious Diseases in Japan, and were propagated and titrated using MDCK cells.

Design of primer sets for RT-LAMP

Primer sets for the RT-LAMP assay were designed using the online LAMP primer design software (PrimerExplorer V4; http://primerexplorer.jp/e/) based on the nucleocapsid protein sequence of the EMC isolate of MERS-CoV (GenBank JX869059.2).

Extraction of nucleic acids

RNA was extracted from viral stocks using TRIzol LS or TRIzol reagent (Invitrogen), according to the manufacturer's instructions. Viral DNA was extracted using Qiagen Genomic-tip (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Total RNA and genomic DNA were quantitated using standard methods to measure the OD value. The MERS-CoV RNA copy number was calculated based upon the standard curve obtained using a TaqMan assay, as described by Corman *et al.* [14] (upE probe set and the positive control template). Total RNA was then diluted with ribonuclease-free water containing 10 μg/mL of Ribonucleic Acid from Baker's Yeast (R6750; Sigma-Aldrich, St. Louis, MO, USA) as carrier RNA.

RT-LAMP assay

The RT-LAMP assay was performed using the Loopamp RNA Amplification Kit (RT-LAMP; Eiken, Tokyo, Japan)

under the following conditions: 5-μL sample (RNA or DNA) was mixed with 40 pmol each of FIP and BIP primers, 20 pmol each of LF and LB primers, 5 pmol each of F3 and B3 primers, 1-μL Enzyme Mix, and 12.5-μL Reaction Mix; distilled water was added to obtain a final volume of 25 μL. For real-time monitoring of RT-LAMP amplification, the reaction mixture was incubated at 65°C for 30 min in a Loopamp real-time turbidimeter (LA-320C, Eiken). For fluorescence detection, 1-μL Fluorescent Detection Reagent (Eiken) was added to the reaction mixture described above before the start of amplification, and then, fluorescence was detected under ultraviolet light after 30 min of amplification. Negative controls containing only yeast RNA were included in each assay.

To synthesize control RNA for RT-LAMP, the nucleocapsid protein sequence of the EMC strain (28566–29807) was amplified and cloned into the pGEM-T easy vector (Promega, Fitchburg, WI, USA). Point mutations were inserted using a site-direct mutagenesis technique to generate variations of the nucleoprotein sequence. The nucleocapsid protein sequence was amplified by PCR using forward (5′-TAATACGACTCACTATAGG GATGGCATCCCCTGCTGCACC-3′) and reverse (5′-CTAATCAGTGTTAACATCAA-3′) primers with PrimeSTAR Max DNA polymerase (Takara-Bio, Shiga, Japan). The amplicons were gel-purified and were used as templates for RNA transcription using a MEGAscript T7 Transcription Kit (Life Technologies, Carlsbad, CA, USA). The transcribed RNA was quantified using the OD value, the copies number was calculated, and the RNA was diluted with ribonuclease-free water containing 10 μg/mL of yeast RNA.

Real-time RT-PCR assays

Real-time RT-PCR assays using upE and ORF1a sets [14,15] were also performed for virus detection using a QuantiTect Probe RT-PCR kit (QIAGEN) and LightCycler 480 Instrument (Roche, Basel, Switzerland) following the manufacturers' protocols. The amplification conditions followed Corman *et al.* [14,15].

Virus preparation for spiked samples

For sensitivity assays, Vero cells were infected with MERS-CoV, and incubated for 4 days. Cell supernatants were then collected and centrifuged at 1,500 × g for 30 min at 4°C, and the supernatants were treated with RNaseA (Nippongene, Tokyo Japan) at a concentration of 10 μg/mL for 30 min at 37°C to exclude miscellaneous RNA other than viral RNA.

Pilot experiment

MERS-CoV RNA was obtained from pre-titrated viral stocks diluted with medium containing pharyngeal swabs obtained from healthy adults using the Universal Viral Transport for Viruses, Chlamydiae, Mycoplasmas and Ureaplasmas (Becton Dickinson and Company, Sparks, MD, USA). Viral isolation was also performed on Vero and Vero/TMPRSS2 cells constitutively expressing type II transmembrane serine protease (TMPRSS2) [30], which enhances cell entry and fusion formation of MERS-CoV [31,32]. Diluted viruses were inoculated on Vero and Vero/TMPRSS2 cells, and incubated for 60 min. Cells were then washed with PBS, and incubated in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum at 37°C. The cytopathic effect was evaluated 5 days after inoculation. Clinical specimens (nasopharyngeal swabs) diagnosed as other respiratory pathogens by RT-PCR assays were used as negative controls. Informed, written consent was obtained at the time of sample collection from all patients. For the specimen diagnosed as human bocavirus, a LAMP assay was performed without the RT reaction.

Results

RT-LAMP primer design

The primer sets used in this study are listed in Table 1. RT-LAMP requires at least six specific sequences (F1, F2, F3, B3, B2, and B1), targeted by a minimum of four distinct primer sets. Two loop primers (LF and LB) are used to enhance amplification [22]; these primers target the regions between F1 & F2, and B1 & B2 regions, respectively. Generally, the reaction of RT-LAMP is performed for 1 h. However, the primers described here tend to generate products of self-construction because these were constructed to enhance the sensitivity of amplification. Therefore, the reaction was performed within 30 min to exclude non-specific reactions. It was confirmed that non-specific amplification did not take place within 30 min using negative control samples (data not shown).

Sensitivity and specificity of the RT-LAMP assay

The detection limit of the RT-LAMP assay was determined using serially diluted MERS-CoV, and compared with those of real-time RT-PCR using upE and ORF1a assays [14,15] (Table 2). upE and ORF1a assays were able to detect 1.6 to 3.4 copies of MERS-CoV RNA, consistent with previous reports. The threshold for RT-LAMP within 30 min was also 3.4 copies, indicating a sensitivity equivalent to that of real-time RT-PCR [14,15].

RT-LAMP amplification can be monitored in three DNA contamination-free manners. First is a real-time method that monitors the turbidity of the pyrophosphate precipitation using a turbidimeter (LA-320C) (Figure 1a). The differentiated value of each signal is calculated automatically at the same time during amplification, with values > 0.1 considered positive. RT-LAMP can also detect MERS-CoV without any specific instruments by detecting visible signals at the same level as real-time

Table 1 The primer set for MERS-CoV RT-LAMP assay

Primers	Position (EMC, JX869059.2)	Sequence (5′ – 3′)	Number of matched MERS sequences*
F3	28848–28866	GCTCCCAGGTGGTACTTCT	86/88
B3	29061–29042	cagtcccctcaatgtggaag	88/88
FIP (F1c + F2)	28939–28918	tcatggacccaaacgatgccatACTGGAACTGGACCCGAAG	77/88
	+ 28872–28890		88/88
BIP (B1c + B2)	28956–28977	GCTCCTTCAACTTTTGGGACGCtagtaccgggcgcgaatt	87/88
	+ 29028–29011		83-88
LF	28906–28891	cggaatgggagtgctg	88/88
LB	28978–29000	GGAACCCTAACAATGATTCAGCT	85/88

Capital letters indicate the sense strand; lowercase letters indicate the antisense strand.
*Eighty-one sequences were obtained from GenBank (JX869059, KJ829365, KJ813439, KJ713299, KJ713298, KJ713297, KJ713296, KJ713295, KJ650297, KJ650296, KJ650295, KJ650098, KJ556336, KJ477102, KJ156953, KJ156952, KJ156949, KJ156944, KJ156942, KJ156939, KJ156934, KJ156932, KJ156927, KJ156917, KJ156916, KJ156913, KJ156911, KJ156910, KJ156909, KJ156907, KJ156906, KJ156905, KJ156902, KJ156901, KJ156883, KJ156881, KJ156876, KJ156874, KJ156873, KJ156872, KJ156871, KJ156869, KJ156866, KJ156865, KJ156862, KJ156861, KF961222, KF961221, KF958702, KF917527, KF811035, KF745068, KF600652, KF600651, KF600647, KF600645, KF600644, KF600643, KF600639, KF600636, KF600635, KF600634, KF600632, KF600630, KF600628, KF600627, KF600623, KF600621, KF600620, KF600613, KF600612, KF192507, KF186567, KF186566, KF186565, KF186564, KC875821, KC776174, KC667074, KC164505, and KJ782550) and seven sequences were obtained online (England2-HPA, http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1317138176202: Jeddah_2014_C7149, C7569, C7770, C8826, C9055, and C9355, http://www.virology-bonn.de/index.php?id=46).

monitoring. Amplification can be determined through visual detection of magnesium pyrophosphate precipitation following completion of the reaction (Figure 1b). Furthermore, amplicons can be detected by means of green fluorescence under ultraviolet light by adding a fluorescence detection reagent to the mixture before the start of amplification (Figure 1c).

In addition to MERS-CoV, specific amplification via RT-LAMP was also tested using various respiratory viruses (Table 3). RT-LAMP was performed using pretitrated viral stocks, as well as clinical specimens previously validated by PCR. MERS-CoV RT-LAMP was specific for MERS-CoV; other respiratory viruses, such as HCoV, SARS-CoV, RSV, influenza, PIV, ADV, and HMPV could not be amplified using the MERS-CoV primers.

Pilot experiments

As MERS-CoV clinical isolates are not available in Japan, a pilot study was performed using MERS-CoV laboratory isolates diluted with medium containing pharyngeal swabs obtained from healthy adults. Viral detection was carried out using both RT-LAMP and real-time RT-PCR assays, and with virus isolation using Vero and Vero/TMPRSSS2 cells (Table 4). Viral isolation from Vero cells required at least 500 copies of MERS-CoV, followed by incubation at 37°C for 5 days. It contrast, although it has been reported that TMPRSS2 enhances the entry and fusion formation of MERS-CoV [31,32], Vero/TMPRSS2 cells exhibited syncytium formation with 23.2 copies of MERS-CoV within 2 days of incubation.

The real-time RT-PCR was highly sensitive for MERS-CoV, with upE and ORF1a assays capable of detecting as few as 1.6 copies of MERS-CoV RNA. The RT-LAMP was also able to detect viral RNA at levels as low as 0.7 copies, showing equivalence with the RT-PCR assay. Viral isolation using Vero/TMPRSS2 cells was more sensitive than that of Vero cells; however, genetic diagnostic assays were consistently more sensitive than culture-based methods. These data suggest that the RT-LAMP assay is capable of detecting MERS-CoV with a sensitivity similar to that of real-time-RT-PCR, even in clinical specimens.

Next, clinical specimens previously diagnosed as other respiratory pathogens were tested using the RT-LAMP assay (Table 5). All reactions were negative, with no cross reactivity for other respiratory viruses, indicating a high degree of specificity for the RT-LAMP assay. Collectively,

Table 2 Sensitivity of the RT-LAMP assay

Copies/reaction	500,000	50,000	5000	500	50	5	0.5	Negative control	Sensitivity (copies)
Real-time RT-PCR*									
upE	21.2	24.4	27.9	31.3	34.2	36.6	> 40	> 40	1.6
ORF1a	21.6	24.4	27.6	30.4	32	31.8	> 40	> 40	3.4
RT-LAMP**	11.33	12.13	13.07	15.44	21.44	22.33	> 30	> 30	3.4

*Threshold cycle.
**Time (min. s).

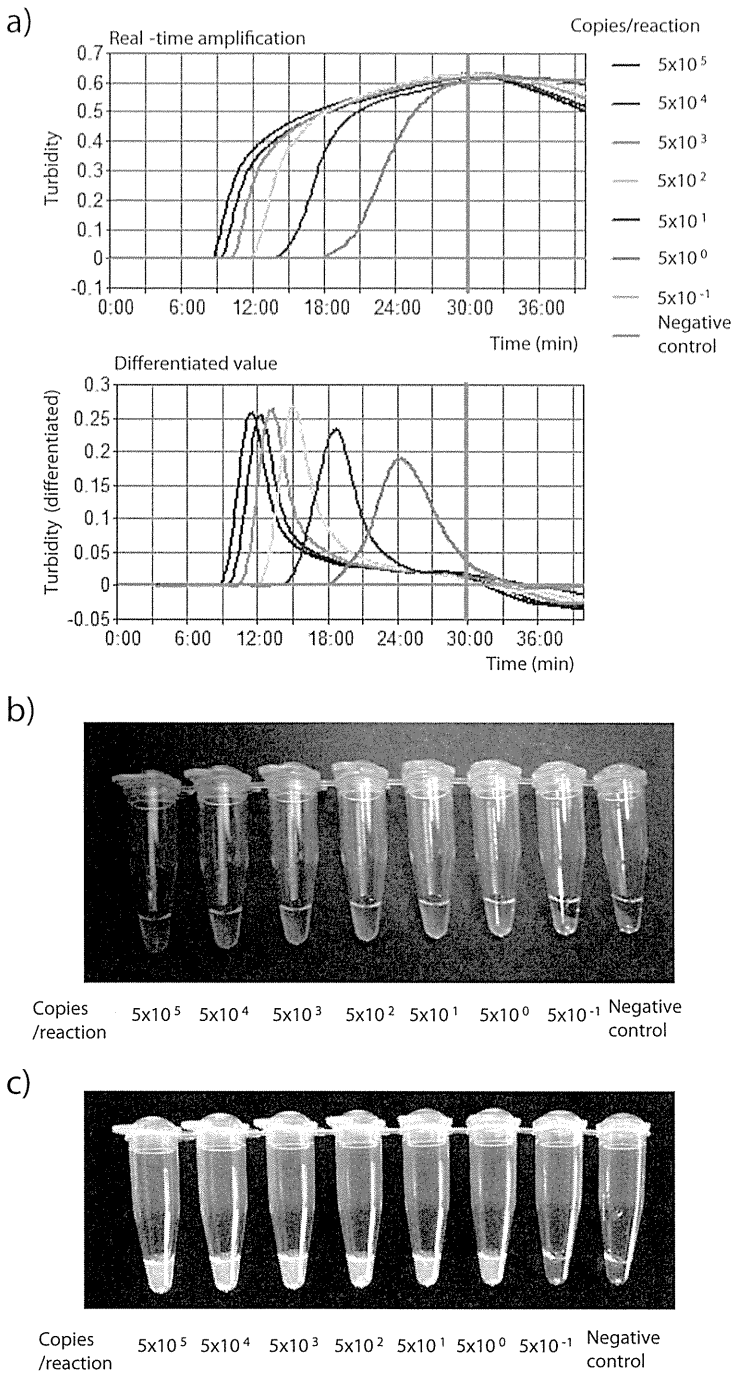

Figure 1 Sensitivity of the MERS-CoV RT-LAMP assay. a) Real-time amplification of MERS-CoV by RT-LAMP. Amplification of serially diluted MERS-CoV RNA was measured in real-time using a Loopamp real-time turbidimeter (LA-320C). The differentiated value at each dilution was calculated automatically, with values > 0.1 within 30 min (red line) considered positive. **b, c)** Detection of RT-LAMP amplicon by **b)** precipitation of magnesium pyrophosphate and **c)** fluorescence under ultra violet light. For fluorescence detection, 1-μL Fluorescence Detection Reagent was added to each reaction mixture. Fluorescent signals were detected under ultraviolet following completion of the amplification reaction. Successful amplification could be detected as green fluorescent light.

Table 3 Specificity of the RT-LAMP assay

Virus	Titer/reaction	Results (min)
Coronaviruses		
MERS-CoV (EMC)	4×10^1 $TCID_{50}$	14.24
HCoV 229E (VR-740)	3×10^3 PFU	> 30
HCoV 229E (Sendai-H/1121/04)	5×10^3 PFU	> 30
HCoV NL63	2.5×10^2 FFU	> 30
HCoV OC43 (VR-1558)	1.3×10^3 $TCID_{50}$	> 30
SARS-CoV (Frankfurt)	1×10^5 PFU	> 30
Other Respiratory Viruses		
RSV A (Long)	1×10^1 PFU	> 30
RSV A (A2)	1×10^2 PFU	> 30
RSV B (18537)	1×10^2 PFU	> 30
RSV B (WV/14617/85)	—*	> 30
HMPV (Sendai-H/2404/2003)	—*	> 30
PIV 1 (C-35)	3×10^4 PFU	> 30
PIV 3 (C-243)	5×10^3 PFU	> 30
ADV 3 (G.B.)	2.5×10^2 $TCID_{50}$	> 30
ADV 4 (RI-67)	1×10^2 $TCID_{50}$	> 30
ADV 7 (Gomen)	2.5×10^2 $TCID_{50}$	> 30
Flu A/California/7/2009 (H1N1pdm)	8×10^3 $TCID_{50}$	> 30
Flu A/Victoria/210/2009 (H3N2)	2.5×10^6 $TCID_{50}$	> 30
Flu B/Brisbane/60/2008	2.5×10^4 $TCID_{50}$	> 30

*Titer unknown, but was confirmed by PCR.
PFU: plaque forming unit.
FFU: focus forming unit.
TCID50: 50% tissue culture infectious dose.

these results suggest that the MERS-CoV RT-LAMP assay is useful for epidemiological surveillance in suspected clinical cases.

RT-LAMP validation for mismatched sequences

The primer set was constructed based on the conserved region of the nucleocapsid protein sequence of the EMC isolate of MERS-CoV (GenBank accession no JX869059.2).

Table 5 Detection of MERS-CoV using clinical specimens diagnosed as other respiratory viral infections

Diagnosed pathogen	Results (min)
Positive control	
*MERS-CoV (10^4 copies)	9.00
Coronaviruses	
HCoV OC43	> 30
HCoV NL63	> 30
HCoV HKU1	> 30
Other Respiratory viruses	
RSV A	> 30
RSV B	> 30
HMPV	> 30
PIV 1	> 30
PIV 2	> 30
PIV 3	> 30
PIV 4	> 30
Rhinovirus	> 30
Bocavirus	> 30
Flu A H1 (Russian)	> 30
Flu A H1 (2009 pdm)	> 30
Flu A H3	> 30
Flu B (Yamagata)	> 30
Flu B (Victoria)	> 30
Flu C	> 30
Measles virus	> 30
Rubella virus	> 30

*Synthesized RNA obtained from N protein region.

The number of sequences deposited in GenBank is increasing, and this region of MERS-CoV exhibits several sequences variation. Therefore, the primer sequences were checked against the available MERS-CoV sequences. Eighty-one MERS-CoV nucleocapsid sequences were collected from GenBank, along with seven other sequences

Table 4 Detection of MERS-CoV diluted with medium containing pharyngeal swabs

Copies/50 μL	500,000	50,000	5000	500	50	5	0.5	Negative control	Sensitivity (copies)	Time required
Virus isolation*										
Vero	6/6	6/6	6/6	3/6	0/6	0/6	0/6	0/6	500	5 d
Vero/TMPRSS2	6/6	6/6	6/6	6/6	5/6	0/6	0/6	0/6	23.2	2 d
Copies/reaction	**50,000**	**5000**	**500**	**50**	**5**	**0.5**	**0.05**			
Real-time RT-PCR**										
upE	22.0	25.3	28.7	32.3	33.2	> 40	> 40	> 40	1.6	2 hr
ORF1a	21.9	25.2	28.5	32.2	32.5	> 40	> 40	> 40	1.6	2 hr
RT-LAMP***	11.00	11.16	12.18	13.48	18.04	23.12	> 30	> 30	0.7	30 min

*Positive number/tested number.
**Threshold cycle.
***Time (min. s).

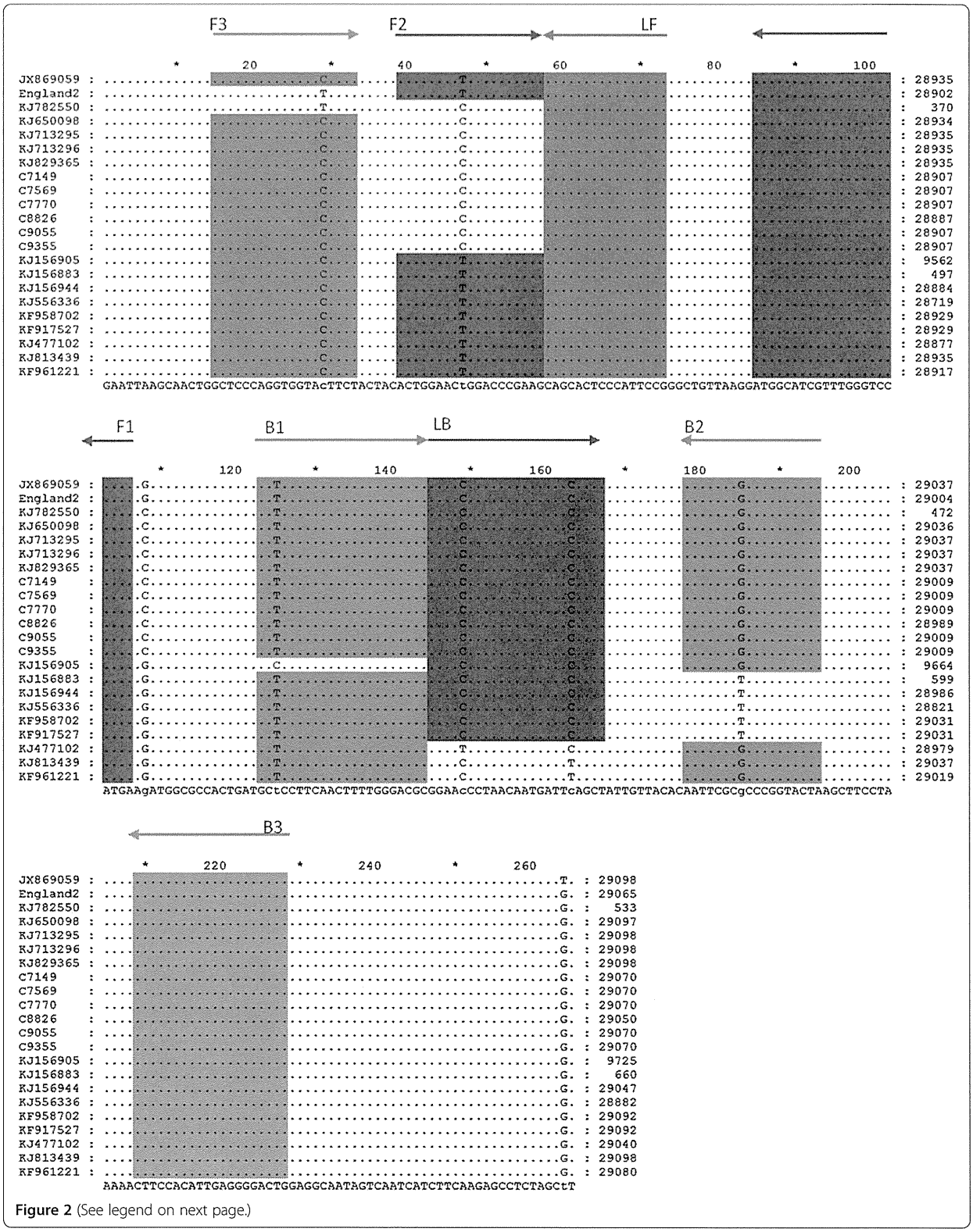

Figure 2 (See legend on next page.)

(See figure on previous page.)
Figure 2 Nucleotide mismatches in the MERS-CoV sequences. The MERS-CoV sequences that have mismatches with the RT-LAMP primer sets were identified in an alignment based on the sequence of the EMC isolate (JX869059.2). The positions of six essential regions (F3, F2, F1, B3, B2, and B1) and loop primers (LF and LB) are indicated. The accession numbers of the MERS-CoV sequences used in the alignment were as follows: JX869059, KJ782550, KJ650098, KJ713295, KJ713296, KJ829365, KJ156905, KJ156883, KJ156944, KJ556336, KF958702, KF917527, KJ477102, KJ813439 and KF961221. Seven MERS-CoV sequences available online (England2, Jeddah_2014_C7149, C7569, C7770, C8826, C9055 and C9355) were also used. The alignment was performed using GeneDoc ver. 2.7 (http://www.nrbsc.org/gfx/genedoc/).

reported online (England2-HPA, Jeddah_2014_C7149, C7569, C7770, C8826, C9055, and C9355]. An alignment was constructed using the EMC isolate and mismatched sequences (Figure 2). The RT-LAMP primers matched most of the 88 sequences. Primers B3 and LF matched them completely. Primer F3 had one nucleotide mismatch for two sequences. Primer LB had one nucleotide mismatch for one sequence and had another mismatch for two sequences. Primer BIP had one nucleotide mismatch in B1 region of one sequence and had another mismatch in the B2 region of five sequences. Primer FIP had a one-nucleotide mismatch in the F1 region of 11 sequences (Table 1). The efficiency of RT-LAMP for these mismatched sequences was evaluated using an RNA template transcribed from a 1242-bp of PCR amplicon from the nucleocapsid gene sequence of MERS-CoV (Table 6). The sensitivity of RT-LAMP using the RNA control template of the EMC isolate was 15.8 copies and tended to be lower than that using viral RNA as the template. This might be caused by a difference in RT efficiency or the secondary structure of RNA due to the difference in length. The mismatches in the F3, F2, B1, and LB region did not markedly affect the amplification efficiency, and the sensitivity was equal to or twice that of the EMC isolate. The mismatch in the B2 region affected amplification slightly, and showed a fivefold decrease in the sensitivity (73.4 copies). This mismatch in B2 was seen in 5 sequences (KJ156883, KJ156944, KF556336, KF958702, and KF917527) of the 88 MERS-CoV sequences checked in this study. Nevertheless, these results suggest that the RT-LAMP primer set facilitates amplification of the remaining 83 sequences to the same level as the EMC isolate.

Discussion

As described above, definitive MERS-CoV diagnosis requires amplification of at least two different virus-specific genomic targets according to the case definition reported on 3 July 2013 by the WHO. However, only two real-time RT-PCR targets, upE and ORF1a, are currently available for MERS-CoV detection with high specificity and sensitivity [14,15]. Indeed, two previous cases reported by the Italian government had to be reclassified as probable MERS-CoV infections, as they were unable to fulfill these criteria (WHO, GAR, MERS-CoV summary and literature update – as of 20 September 2013, http://www.who.int/csr/disease/coronavirus_infections/update_20130920/en/index.html). Additional sensitive and specific genetic diagnostic methods are therefore needed to provide reliable MERS-CoV diagnoses.

This study describes a novel genetic diagnostic method for MERS-CoV based on the RT-LAMP assay, with a sensitivity and specificity equal to that of the upE and ORF1a RT-PCR assays. This assay was also able to detect MERS-CoV RNA in experimentally obtained nasopharyngeal swabs, and never showed cross reactivity to other respiratory viruses, even in the case of clinical specimens.

The RT-LAMP method requires only a single temperature for amplification, with results usually available in less than 1 h by observing magnesium pyrophosphate

Table 6 Evaluation of RT-LAMP amplification using mismatched sequences

Accession	Name				Sensitivity (copies)
JX869059	EMC				15.8
Representative sequence		**Nucleotide position***	**Substitution**	**Region in primer**	**Sensitivity (copies)**
	England2	28862	C to T	F3	7.3
KJ782550	Greece-Saudi Arabia_2014	28862	C to T	F3	15.8
		28880	T to C	FIP (F2)	
KJ650098	Camel/Qatar_2_2014	28880	T to C	FIP (F2)	34.1
KJ156905	Riyadh_7b_2013	28958	T to C	BIP (B1)	15.8
KF917527	Jeddah-Camel-1	29018	G to T	BIP (B2)	73.4
KJ477102	NRCE-HKU205	28982	C to T	LB	39.7
KF961221	Qatar3	28996	C to T	LB	7.3

*Based on EMC isolate (JX869059.2).

precipitate or fluorescence signals by the naked eye [21,22]. Although RT-LAMP amplification can be monitored in real-time using a turbidimeter [23], the assay can also be performed using basic laboratory equipment, such as a heat block and water bath. The method has been validated using various respiratory viruses, as well as more diverse pathogens, such as bacteria [33-35], protozoa [36-38], and parasites [39,40]. Furthermore, the reagents necessary to perform RT-LAMP are commercially available. Recently, Abd El Wahed et al., reported a genetic diagnostic method for MERS-CoV based on reverse transcription isothermal recombinase polymerase amplification (RT-PPA) assay. The method is highly sensitive and can detect 10 copies of virus RNA within 3 to 7 minutes. Therefore, it is useful for the diagnosis of MERS-CoV as well. However, RT-RPA requires a specific tubescanner for detection. By contrast, the RT-LAMP assay enables detection of amplicons by observing a magnesium pyrophosphate precipitate or fluorescence signals with the naked eye with no requirement for any specialized instruments. Taken together, the specificity and sensitivity of the RT-LAMP assay described here, in combination with its accessibility and ease of use, make this assay a valuable tool for the diagnosis and epidemiologic surveillance of human MERS-CoV infection, especially for field use.

The primer sets described in this study matched to most of the available sequences. However, several sequences had mismatches with the primers. The effect of these mismatches on the RT-LAMP efficiency was evaluated using a synthesized control RNA template: most of the mismatches did not affect the amplification efficiency of RT-LAMP. However, the substitution in B2 region of the primer, namely, a G to T substitution at position 29018 in the nucleocapsid gene sequence of the EMC isolate, was present in five MERS-CoV sequences and caused a slight decrease in sensitivity. Of the five sequences, KJ156883 (Asir_1_2013) belongs to the Buraidah_1 clade, and is the only sequence in this clade with such a substitution [41]. The other sequences (KJ156944, Riyadh_5_2013; KJ556336, Jeddah_1_2013; KF958702, Jeddah_human1; KF917527, Jeddah_Camel1) belong to the Riyadh_3 clade [42,43]; none of the other sequences in this clade have a substitution according to the alignment analysis. Therefore, the substitution does not represent a major population in this clade. Although it is important to improve the primers to increase their sensitivity for sequences in the Riyadh_3 clade by using mixed bases, the results of this study suggest that the RT-LAMP primer is useful for detecting the majority of the prevalent MERS-CoV strains.

The success rate of MERS-CoV detection is dependent on the collection of clinical specimens. Drosten *et al.*, reported that up to 10^6 copies/mL of MERS-CoV RNA are present in lower respiratory tract specimens, such as tracheobronchial secretions, and bronchoalveolar lavage. In contrast, only small amounts of viral RNAs were detected in upper respiratory tract specimens and other tissue [44]. The RT-LAMP primers described here were designed based upon the nucleocapsid protein sequence of MERS-CoV, due to the unique coronaviral replication system. Although coronaviruses generate subgenomic mRNAs to produce each viral protein, all subgenomic mRNAs contain nucleoprotein sequences, as they are located on the 3′end of the coronavirus genome [45-47]. This implies that the RT-LAMP procedure described here will exhibit a high degree of sensitivity for specimens containing cellular components.

Conclusions

This study developed a RT-LAMP assay for the MERS-CoV, which was capable of detecting as few as 3.4 copies of MERS-CoV RNA, and was highly specific, with no cross-reaction with other respiratory viruses. These results suggest that the RT-LAMP assay described here is a useful tool for the diagnosis and epidemiologic surveillance of human MERS-CoV infections.

Abbreviations

CoV: Coronavirus; FFU: Focus forming unit; MERS: Middle East respiratory syndrome; ORF: Open reading frame; PBS: Phosphate-buffered saline; PFU: Plaque forming unit; RT-LAMP: Reverse transcription-loop-mediated isothermal amplification; TCID50: 50% tissue culture infectious dose; TMPRSS2: Transmembrane protease, serine 2; upE: Upstream E.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors participating in the planning of the project. SS and TsuN constructed the RT-LAMP primer set and evaluated the sensitivity. TY, SA, TK, and TaN participated in the pilot experiments. KS participated in all experiments and wrote manuscript. SM is the leader of the project. All authors read and approved the final manuscript.

Acknowledgements

We thank Dr. Ron A. M. Fouchier, Erasmus Medical Center, Rotterdam, The Netherlands for providing MERS-CoV EMC isolate. We also thank the staff of Mie Prefecture Health and Environment Research Institute, Mie, Japan. This work was supported by a grant-in-aid from the Ministry of Health, Labor, and Welfare, Japan. We thank Dr. Aiko Fukuma and Dr. Shuetsu Fukushi, Department of Virology I, National Institute of Infectious Diseases, Japan, for cloning of the nucleocapsid sequence of MERS-CoV.

Author details

[1]Laboratory of Acute Respiratory Viral Diseases and Cytokines, Department of Virology III, National Institute of Infectious Disease, Laboratory of Acute Respiratory Viral Diseases and Cytokines, 4-7-1 Gakuen, Musashimurayama, Tokyo 208-0011, Japan. [2]Mie Prefecture Health and Environment Research Institute, 3684-11 Sakura-cho, Yokkaichi, Mie 512-1211, Japan. [3]Eiken Chemical Co. Ltd., 4-19-9 Taito, Taito-ku, Tokyo 110-8408, Japan.

Received: 25 April 2014 Accepted: 4 August 2014
Published: 8 August 2014

References

1. Danielsson N, On Behalf Of The Ecdc Internal Response Team C, Catchpole M: **Novel coronavirus associated with severe respiratory disease: case definition and public health measures.** *Euro Surveill* 2012, **17**:20282.
2. Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus AD, Fouchier RA: **Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia.** *N Engl J Med* 2012, **367**:1814–1820.
3. de Groot RJ, Baker SC, Baric RS, Brown CS, Drosten C, Enjuanes L, Fouchier RA, Galiano M, Gorbalenya AE, Memish ZA, Perlman S, Poon LL, Snijder EJ, Stephens GM, Woo PC, Zaki AM, Zambon M, Ziebuhr J: **Middle East respiratory syndrome coronavirus (MERS-CoV): announcement of the coronavirus study group.** *J Virol* 2013, **87**:7790–7792.
4. van Boheemen S, de Graaf M, Lauber C, Bestebroer TM, Raj VS, Zaki AM, Osterhaus AD, Haagmans BL, Gorbalenya AE, Snijder EJ, Fouchier RA: **Genomic characterization of a newly discovered coronavirus associated with acute respiratory distress syndrome in humans.** *MBio* 2012, **3**:e00473–00412.
5. Peiris JS, Guan Y, Yuen KY: **Severe acute respiratory syndrome.** *Nat Med* 2004, **10**:S88–97.
6. Li W, Shi Z, Yu M, Ren W, Smith C, Epstein JH, Wang H, Crameri G, Hu Z, Zhang H, Zhang J, McEachern J, Field H, Daszak P, Eaton BT, Zhang S, Wang LF: **Bats are natural reservoirs of SARS-like coronaviruses.** *Science* 2005, **310**:676–679.
7. Lau SK, Li KS, Huang Y, Shek CT, Tse H, Wang M, Choi GK, Xu H, Lam CS, Guo R, Chan KH, Zheng BJ, Woo PC, Yuen KY: **Ecoepidemiology and complete genome comparison of different strains of severe acute respiratory syndrome-related rhinolophus bat coronavirus in China reveal bats as a reservoir for acute, self-limiting infection that allows recombination events.** *J Virol* 2010, **84**:2808–2819.
8. Hemida MG, Perera RA, Wang P, Alhammadi MA, Siu LY, Li M, Poon LL, Saif L, Alnaeem A, Peiris M: **Middle East respiratory syndrome (MERS) coronavirus seroprevalence in domestic livestock in Saudi Arabia, 2010 to 2013.** *Euro Surveill* 2013, **18**:20659.
9. Kupferschmidt K: **Emerging diseases: researchers scramble to understand camel connection to MERS.** *Science* 2013, **341**:702.
10. Reusken C, Ababneh M, Raj V, Meyer B, Eljarah A, Abutarbush S, Godeke G, Bestebroer T, Zutt I, Muller M, Bosch B, Rottier P, Osterhaus A, Drosten C, Haagmans B, Koopmans M: **Middle East respiratory syndrome coronavirus (MERS-CoV) serology in major livestock species in an affected region in Jordan, June to September 2013.** *Euro Surveill* 2013, **18**:20662.
11. Reusken CB, Haagmans BL, Muller MA, Gutierrez C, Godeke GJ, Meyer B, Muth D, Raj VS, Smits-De Vries L, Corman VM, Drexler JF, Smits SL, El Tahir YE, De Sousa R, van Beek J, Nowotny N, van Maanen K, Hidalgo-Hermoso E, Bosch BJ, Rottier P, Osterhaus A, Gortazar-Schmidt C, Drosten C, Koopmans MP: **Middle East respiratory syndrome coronavirus neutralising serum antibodies in dromedary camels: a comparative serological study.** *Lancet Infect Dis* 2013, **13**:859–866.
12. Alagaili AN, Briese T, Mishra N, Kapoor V, Sameroff SC, Burbelo PD, de Wit E, Munster VJ, Hensley LE, Zalmout IS, Kapoor A, Epstein JH, Karesh WB, Daszak P, Mohammed OB, Lipkin WI: **Middle East respiratory syndrome coronavirus infection in dromedary camels in Saudi Arabia.** *MBio* 2014, **5**:e00884–00814.
13. Haagmans BL, Al Dhahiry SH, Reusken CB, Raj VS, Galiano M, Myers R, Godeke GJ, Jonges M, Farag E, Diab A, Ghobashy H, Alhajri F, Al-Thani M, Al-Marri SA, Al Romaihi HE, Al Khal A, Bermingham A, Osterhaus AD, AlHajri MM, Koopmans MP: **Middle East respiratory syndrome coronavirus in dromedary camels: an outbreak investigation.** *Lancet Infect Dis* 2014, **14**:140–145.
14. Corman V, Eckerle I, Bleicker T, Zaki A, Landt O, Eschbach-Bludau M, van Boheemen S, Gopal R, Ballhause M, Bestebroer T, Muth D, Muller M, Drexler J, Zambon M, Osterhaus A, Fouchier R, Drosten C: **Detection of a novel human coronavirus by real-time reverse-transcription polymerase chain reaction.** *Euro Surveill* 2012, **17**:20285.
15. Corman VM, Muller MA, Costabel U, Timm J, Binger T, Meyer B, Kreher P, Lattwein E, Eschbach-Bludau M, Nitsche A, Bleicker T, Landt O, Schweiger B, Drexler JF, Osterhaus AD, Haagmans BL, Dittmer U, Bonin F, Wolff T, Drosten C: **Assays for laboratory confirmation of novel human coronavirus (hCoV-EMC) infections.** *Euro Surveill* 2012, **17**:20334.
16. Chen W, He B, Li C, Zhang X, Wu W, Yin X, Fan B, Fan X, Wang J: **Real-time RT-PCR for H5N1 avian influenza A virus detection.** *J Med Microbiol* 2007, **56**:603–607.
17. Pang X, Lee B, Chui L, Preiksaitis JK, Monroe SS: **Evaluation and validation of real-time reverse transcription-pcr assay using the LightCycler system for detection and quantitation of norovirus.** *J Clin Microbiol* 2004, **42**:4679–4685.
18. Ke GM, Cheng HL, Ke LY, Ji WT, Chulu JL, Liao MH, Chang TJ, Liu HJ: **Development of a quantitative light cycler real-time RT-PCR for detection of avian reovirus.** *J Virol Methods* 2006, **133**:6–13.
19. Yan L, Yan P, Zhou J, Teng Q, Li Z: **Establishing a TaqMan-based real-time PCR assay for the rapid detection and quantification of the newly emerged duck Tembusu virus.** *Virol J* 2011, **8**:464.
20. Parida MM: **Rapid and real-time detection technologies for emerging viruses of biomedical importance.** *J Biosci* 2008, **33**:617–628.
21. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T: **Loop-mediated isothermal amplification of DNA.** *Nucleic Acids Res* 2000, **28**:E63.
22. Nagamine K, Hase T, Notomi T: **Accelerated reaction by loop-mediated isothermal amplification using loop primers.** *Mol Cell Probes* 2002, **16**:223–229.
23. Mori Y, Nagamine K, Tomita N, Notomi T: **Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation.** *Biochem Biophys Res Commun* 2001, **289**:150–154.
24. Hong TC, Mai QL, Cuong DV, Parida M, Minekawa H, Notomi T, Hasebe F, Morita K: **Development and evaluation of a novel loop-mediated isothermal amplification method for rapid detection of severe acute respiratory syndrome coronavirus.** *J Clin Microbiol* 2004, **42**:1956–1961.
25. Shirato K, Nishimura H, Saijo M, Okamoto M, Noda M, Tashiro M, Taguchi F: **Diagnosis of human respiratory syncytial virus infection using reverse transcription loop-mediated isothermal amplification.** *J Virol Methods* 2007, **139**:78–84.
26. Ushio M, Yui I, Yoshida N, Fujino M, Yonekawa T, Ota Y, Notomi T, Nakayama T: **Detection of respiratory syncytial virus genome by subgroups-A, B specific reverse transcription loop-mediated isothermal amplification (RT-LAMP).** *J Med Virol* 2005, **77**:121–127.
27. Imai M, Ninomiya A, Minekawa H, Notomi T, Ishizaki T, Tashiro M, Odagiri T: **Development of H5-RT-LAMP (loop-mediated isothermal amplification) system for rapid diagnosis of H5 avian influenza virus infection.** *Vaccine* 2006, **24**:6679–6682.
28. Mahony J, Chong S, Bulir D, Ruyter A, Mwawasi K, Waltho D: **Multiplex loop-mediated isothermal amplification (M-LAMP) assay for the detection of influenza A/H1, A/H3 and influenza B can provide a specimen-to-result diagnosis in 40 min with single genome copy sensitivity.** *J Clin Virol* 2013, **58**:127–131.
29. Shirato K, Kawase M, Watanabe O, Hirokawa C, Matsuyama S, Nishimura H, Taguchi F: **Differences in neutralizing antigenicity between laboratory and clinical isolates of HCoV-229E isolated in Japan in 2004–2008 depend on the S1 region sequence of the spike protein.** *J Gen Virol* 2012, **93**:1908–1917.
30. Shirogane Y, Takeda M, Iwasaki M, Ishiguro N, Takeuchi H, Nakatsu Y, Tahara M, Kikuta H, Yanagi Y: **Efficient multiplication of human metapneumovirus in Vero cells expressing the transmembrane serine protease TMPRSS2.** *J Virol* 2008, **82**:8942–8946.
31. Gierer S, Bertram S, Kaup F, Wrensch F, Heurich A, Kramer-Kuhl A, Welsch K, Winkler M, Meyer B, Drosten C, Dittmer U, von Hahn T, Simmons G, Hofmann H, Pohlmann S: **The spike protein of the emerging betacoronavirus EMC uses a novel coronavirus receptor for entry, can be activated by TMPRSS2, and is targeted by neutralizing antibodies.** *J Virol* 2013, **87**:5502–5511.
32. Shirato K, Kawase M, Matsuyama S: **Middle East respiratory syndrome coronavirus infection mediated by the transmembrane serine protease TMPRSS2.** *J Virol* 2013, **87**:12552–12561.
33. Ueda S, Kuwabara Y: **The rapid detection of Salmonella from food samples by loop-mediated isothermal amplification (LAMP).** *Biocontrol Sci* 2009, **14**:73–76.
34. Geojith G, Dhanasekaran S, Chandran SP, Kenneth J: **Efficacy of loop mediated isothermal amplification (LAMP) assay for the laboratory identification of Mycobacterium tuberculosis isolates in a resource limited setting.** *J Microbiol Methods* 2011, **84**:71–73.
35. Gotoh K, Nishimura N, Ohshima Y, Arakawa Y, Hosono H, Yamamoto Y, Iwata Y, Nakane K, Funahashi K, Ozaki T: **Detection of Mycoplasma pneumoniae by loop-mediated isothermal amplification (LAMP) assay**

and serology in pediatric community-acquired pneumonia. *J Infect Chemother* 2012, 18:662–667.
36. Plutzer J, Karanis P: Rapid identification of Giardia duodenalis by loop-mediated isothermal amplification (LAMP) from faecal and environmental samples and comparative findings by PCR and real-time PCR methods. *Parasitol Res* 2009, 104:1527–1533.
37. He L, Zhou YQ, Oosthuizen MC, Zhao JL: Loop-mediated isothermal amplification (LAMP) detection of Babesia orientalis in water buffalo (Bubalus babalis, Linnaeus, 1758) in China. *Vet Parasitol* 2009, 165:36–40.
38. Wang LX, He L, Fang R, Song QQ, Tu P, Jenkins A, Zhou YQ, Zhao JL: Loop-mediated isothermal amplification (LAMP) assay for detection of Theileria sergenti infection targeting the p33 gene. *Vet Parasitol* 2010, 171:159–162.
39. Nakao R, Stromdahl EY, Magona JW, Faburay B, Namangala B, Malele I, Inoue N, Geysen D, Kajino K, Jongejan F, Sugimoto C: Development of loop-mediated isothermal amplification (LAMP) assays for rapid detection of Ehrlichia ruminantium. *BMC Microbiol* 2010, 10:296.
40. Arimatsu Y, Kaewkes S, Laha T, Hong SJ, Sripa B: Rapid detection of Opisthorchis viverrini copro-DNA using loop-mediated isothermal amplification (LAMP). *Parasitol Int* 2012, 61:178–182.
41. Cotten M, Watson SJ, Zumla AI, Makhdoom HQ, Palser AL, Ong SH, Al Rabeeah AA, Alhakeem RF, Assiri A, Al-Tawfiq JA, Albarrak A, Barry M, Shibl A, Alrabiah FA, Hajjar S, Balkhy HH, Flemban H, Rambaut A, Kellam P, Memish ZA: Spread, circulation, and evolution of the Middle East respiratory syndrome coronavirus. *MBio* 2014, 5:e01062-01013.
42. Azhar EI, El-Kafrawy SA, Farraj SA, Hassan AM, Al-Saeed MS, Hashem AM, Madani TA: Evidence for camel-to-human transmission of MERS coronavirus. *N Engl J Med* 2014, 370:2499–2505.
43. Memish ZA, Cotten M, Meyer B, Watson SJ, Alsahafi AJ, Al Rabeeah AA, Corman VM, Sieberg A, Makhdoom HQ, Assiri A, Al Masri M, Aldabbagh S, Bosch BJ, Beer M, Muller MA, Kellam P, Drosten C: Human infection with MERS coronavirus after exposure to infected camels, Saudi Arabia, 2013. *Emerg Infect Dis* 2014, 20:1012–1015.
44. Drosten C, Seilmaier M, Corman VM, Hartmann W, Scheible G, Sack S, Guggemos W, Kallies R, Muth D, Junglen S, Muller MA, Haas W, Guberina H, Rohnisch T, Schmid-Wendtner M, Aldabbagh S, Dittmer U, Gold H, Graf P, Bonin F, Rambaut A, Wendtner CM: Clinical features and virological analysis of a case of Middle East respiratory syndrome coronavirus infection. *Lancet Infect Dis* 2013, 13:745–751.
45. Baric RS, Stohlman SA, Lai MM: Characterization of replicative intermediate RNA of mouse hepatitis virus: presence of leader RNA sequences on nascent chains. *J Virol* 1983, 48:633–640.
46. Lai MM, Patton CD, Baric RS, Stohlman SA: Presence of leader sequences in the mRNA of mouse hepatitis virus. *J Virol* 1983, 46:1027–1033.
47. Spaan WJ, Rottier PJ, Horzinek MC, van der Zeijst BA: Sequence relationships between the genome and the intracellular RNA species 1, 3, 6, and 7 of mouse hepatitis virus strain A59. *J Virol* 1982, 42:432–439.

doi:10.1186/1743-422X-11-139
Cite this article as: Shirato *et al.*: Detection of Middle East respiratory syndrome coronavirus using reverse transcription loop-mediated isothermal amplification (RT-LAMP). *Virology Journal* 2014 11:139.

Jpn. J. Infect. Dis., 67, 469-475, 2014

Original Article

Associations between Co-Detected Respiratory Viruses in Children with Acute Respiratory Infections

Atsushi Kaida[1]*, Hideyuki Kubo[1], Koh-ichi Takakura[1], Jun-ichiro Sekiguchi[1], Seiji P. Yamamoto[1], Urara Kohdera[2], Masao Togawa[3], Kiyoko Amo[3], Masashi Shiomi[4], Minori Ohyama[1], Kaoru Goto[1], Atsushi Hase[1], Tsutomu Kageyama[5], and Nobuhiro Iritani[1]

[1]*Department of Microbiology, Osaka City Institute of Public Health and Environmental Sciences, Osaka 543-0026;*
[2]*Nakano Children's Hospital, Osaka 535-0022;*
[3]*Osaka City General Hospital, Osaka 534-0021;*
[4]*Aizenbashi Hospital, Osaka 556-0005; and*
[5]*Influenza Virus Research Center, National Institute of Infectious Diseases, Tokyo 208-0011, Japan*

(Received February 13, 2014. Accepted April 30, 2014)

SUMMARY: Viruses are the major etiological agents of acute respiratory infections (ARIs) in young children. Although respiratory virus co-detections are common, analysis of combinations of co-detected viruses has never been conducted in Japan. Nineteen respiratory viruses or subtypes were surveyed using multiplex real-time PCR on 1,044 pediatric (patient age < 6 years) ARI specimens collected in Osaka City, Japan between January 2010 and December 2011. In total, 891 specimens (85.3%) were virus positive (1,414 viruses were detected), and 388 of the virus-positive specimens (43.5%, 388/891) were positive for multiple viruses. The ratio of multiple/total respiratory virus-positive specimens was high in children aged 0–35 months. Statistical analyses revealed that human bocavirus 1 and human adenovirus were synchronously co-detected. On the other hand, co-detections of human parainfluenza virus type 1 (HPIV-1) with HPIV-3, HPIV-3 with human metapneumovirus (hMPV), hMPV with respiratory syncytial virus A (RSV A), hMPV with influenza virus A (H1N1) 2009 (FLUA (H1N1) 2009), RSV A with RSV B, and human rhinovirus and FLUA (H1N1) 2009 were exclusive. These results suggest that young children (<3 years) are highly susceptible to respiratory viruses, and some combinations of viruses are synchronously or exclusively co-detected.

INTRODUCTION

More than 200 viruses have been identified as major etiological agents of acute respiratory infections (ARIs) (1,2). Respiratory virus co-detections in clinical specimens have been reported in ARI patients from infants to adults (3–5). The effects of virus co-detections on clinical severity have been analyzed (6–9). Although some reports have analyzed pairs of co-detected respiratory viruses throughout the world, no analysis has been performed in Japan (10–13). The purpose of this study was to survey multiple respiratory viruses in young children with ARIs; to examine the prevalence of co-detections in virus-positive specimens; to examine the seasonality of virus co-detections; to analyze the combinations of co-detected respiratory viruses; and to examine the ratio of multiple/total virus-positive specimens in different age groups.

Virus isolation tests in cultured cells have been the gold standard method for detecting viruses in clinical specimens. However, some respiratory viruses such as human bocavirus 1 (HBoV 1) and human coronavirus (HCoV) HKU1 do not produce clear cytopathic effects in cultured cells (14,15). Indeed, their discovery was dependent on molecular analyses (14,15). Gene amplification is generally regarded as a highly sensitive and specific method compared with virus isolation using cultured cells (16,17). Multiplex PCR can be used for the simultaneous detection of multiple pathogens in clinical specimens. This study was performed using multiplex real-time PCR to detect multiple respiratory viruses in clinical specimens from young children with ARI over a period of 2 years.

*Corresponding author: Mailing address: Department of Microbiology, Osaka City Institute of Public Health and Environmental Sciences, 8-34 Tojo-cho, Tennoji-ku, Osaka 543-0026, Japan. Tel: +81-6-6771-3147, Fax: +81-6-6772-0676, E-mail: a-kaida@city.osaka.lg.jp

MATERIALS AND METHODS

Clinical specimens: Between January 2010 and December 2011, 1,044 specimens (844 nasal mucus, 121 throat swab, 54 sputum, 15 mouth wash, and 10 tracheal aspirate specimens) were obtained from children (<6 years of age) with suspected viral ARI. Specimens were collected from 6 hospitals/clinics at the discretion of the treating physician and were subsequently used in molecular analyses as part of the passive surveillance program initiated in Osaka City, Japan, which includes ethical consideration (18). The patients (male, 588; female, 456) enrolled in this study were aged between 0–71 months (median age, 15 months; interquartile range, 9–27 months). Most specimens (82.5%) were obtained from young children of <36 months.

Molecular analysis: Viral nucleic acid was extracted using the QIAamp Viral RNA mini kit (Qiagen, Hilden,

Germany), which is useful for the simultaneous extraction of RNA and DNA (19). cDNA was synthesized with random hexamer primers using SuperScript III (Life Technologies, Tokyo, Japan) as described previously (18). Multiplex real-time PCR was conducted using the QuantiTect multiplex PCR kit (Qiagen) for multiple viruses, including human metapneumovirus (hMPV), respiratory syncytial virus (RSV) A, B, human parainfluenza virus (HPIV) types 1–4, HBoV 1, HCoV (229E, OC43, HKU1, NL63), influenza virus (A [FLUAV], A (H1N1) 2009 [FLUA (H1N1) 2009], B [FLUBV], C [FLUCV]), human adenovirus (HAdV), human enterovirus (HEV), and human rhinovirus (HRV). Sequence information for the primers and probes and the combinations used to detect viruses are shown in Table 1 (18,20–25). The detection limit of the established real-time PCR system was 5–500 copies of virus gene. The PCR conditions were as reported previously (18). In addition, 2 independent methods (real-time PCR and conventional RT-PCR) were used to confirm the detection of HRV and HEV (26).

Statistical analysis: Randomization tests were conducted to determine whether given pairs of viruses infected hosts synchronously or exclusively (27) and were flexibly applied to test the significance of observations. The test repeatedly produced simulations of epidemic case numbers of co-detection under the null hypothesis that infections with 2 viruses were independent. To correct for seasonal bias and to avoid type I errors, data were stratified by a quarterly period, and calculations were separately applied to each. Likely density distributions of co-detection cases were produced for each quarter. The distribution of all co-detections throughout the 2-year study period was then calculated as the sum of stratified co-detections. Significant differences in co-detection rates were identified by comparison with this expected distribution. In this study, 100,000 trials were performed using the Monte Carlo method. Confidence intervals with probability P were defined as intervals between percentiles that corresponded with probability $P/2$ and $1\text{-}P/2$ (2-sided estimation). When intervals did not include the observed numbers of multiple infections, the null hypothesis was rejected and cases of multiple infections were regarded as significantly less frequent. Adjustment of significance levels for multiple comparisons was not applied to tests for frequencies of multiple virus infections. These calculations were performed using R 2.15.1 (28).

RESULTS

Detection of multiple respiratory viruses in clinical specimens: Of the 1,044 specimens analyzed, 891 (85.3%) were virus positive (including 1,414 virus infections), and 388 of these (43.5%, 388/891) were positive for multiple virus infections.

Monthly distribution of individual virus detections and ratios of multiple/total respiratory virus infections: The numbers of individual virus detections per month are shown in Table 2. The most commonly detected virus was HRV (n = 390, 27.6% of all viruses detected), followed by HBoV 1 (n = 176) and HAdV (n = 158). When RSV A (n = 118) and B (n = 80) detections were grouped together, RSV (n = 198) was the second most commonly detected virus.

The following viruses were detected in increasing numbers: HBoV 1, April–June; HPIV-3, April–June; hMPV, March–May; HRV, April–December; FLUAV and FLUA (H1N1) 2009, January–March; and RSV, July–December. Co-detection of another virus was observed in 50% or less of FLUAV, FLUA (H1N1) 2009, FLUBV, HPIV-1, and HPIV-2. The other viruses showed more than 50% in terms of the ratio of multiple/total respiratory virus infections, and among them, HBoV 1, HCoV (NL63, HKU1, 229E), and FLUCV showed more than 80%.

Seasonality of multiple virus-positive specimens: The monthly distributions of single, dual, and ≥triple virus infections are shown in Fig. 1. The virus detection rates in all tested samples were higher than 80%, except between August 2010 and September–December 2011. The proportion of multiple/total virus-positive specimens showed no clear seasonality in this study but appeared to peak in April 2010 and May 2011, which is springtime in Japan.

Proportions of multiple respiratory virus infections among age groups: The virus detection rates in clinical specimens and ratios of multiple/total respiratory virus infections among children of different age groups (0–5, 6–11, 12–23, 24–35, 36–47, 48–59, and 60–71 months) are shown in Fig. 2. The virus detection rate in clinical specimens and ratios of multiple/total respiratory virus infections were as follows: 0–5 months (virus positivity rate, 78.3%; ratio of multiple/total respiratory virus infections, 36.5%), 6–11 (90.0%, 51.5%), 12–23 (90.4%, 52.8%), 24–35 (85.3%, 40.3%), 36–47 (76.7%, 30.3%), 48–59 (80.7%, 17.4%), and 60–71 (77.5%, 22.6%).

The virus positivity rate in clinical specimens was 76.7–80.7% in the 0–5, 36–47, 48–59, and 60–71 month age groups and increased to approximately 85.3–90.4% in the 6–11, 12–23, and 24–35 month age groups. The ratio of multiple/total respiratory virus infections in clinical specimens peaked at 51.5–52.8% in the 6–11 and 12–23 month age groups and was 36.5–40.3% in the 0–5 and 24–35 month age groups. In contrast, children aged ≥36 months had proportionately fewer multi-viral infections (17.4–30.3%).

Synchronous or exclusive co-detection of respiratory viruses: The details of co-detections of 2, 3, or more viruses are shown in Table 3. For dual co-detections, virus combinations of HPIV-3 with HBoV 1; HRV with HAdV, HBoV 1, HPIV-3, hMPV, or RSV (A, B); and HAdV with RSV B were most frequent. Triple or more co-detected viruses were most commonly detected as follows: HAdV with HBoV 1, HPIV-3, or RSV (A, B); HBoV 1 with HPIV-3; and HRV with HAdV, HBoV 1, HPIV-3, hMPV, or RSV A.

The associations between co-detected viruses were statistically analyzed. Significant exclusive co-detection relations were found for the combinations of HPIV-1 and HPIV-3 (randomization test, P = 0.0056), HPIV-3 and hMPV ($P < 0.0001$), hMPV and RSV A ($P < 0.0001$), hMPV and FLUA (H1N1) 2009 (P = 0.0001), RSV A and RSV B (P = 0.0002), and HRV and FLUA (H1N1) 2009 (P = 0.0016). In contrast, a significant synchronous co-detection relation was found between HAdV and HBoV 1 (P = 0.001). No other combinations of viruses were significantly synchronously or ex-

Table 1. Sequence information on primers and probes in multiplex real-time PCR

Set	Virus (Target gene)	Name	Sequence, 5′→3′	Nucleotide position	GenBank no.	Reference
1	hMPV	hMPV F	ARYTGCCRATCTTTGGBGTYATAG	3866-3889	AF371337	(20)
	(Fusion)	hMPV R	TYTKACAATACCAYCCTTGRTCYTC	3988-3964		
		hMPV Probe	(FAM) MAARGCAGCYCCYTCTTGYTCMGRA (BHQ-1)	3909-3933		
	RSV B	RSV B F	AAGATGCAAATCATAAATTCACAGGA	1303-1328	AF013254	(23)
	(Nucleoprotein)	RSV B R	TGATATCCAGCATCTTTAAGTATCTTTATAGTG	1405-1373		Modified
		RSV B Probe	(VIC) CTGGACATAGCATATAAC (MGB)	1357-1340		
	RSV A	RSV A F	AGATCAACTTCTGTCATCCAGCAA	1175-1198	JX069802	(23)
	(Nucleoprotein)	RSV A R	TTCTGCACATCATAATTAGGAGTATCAAT	1258-1230		Modified
		RSV A Probe	(Cy5) CACCATCCAACGGAGCACAGGAGAT (BHQ-3)	1202-1226		
2	HPIV-3	HPIV-3 F	ATGGACATGGCATAATGTGCTAT	8149-8171	FJ455842	Designed
	(HN)	HPIV-3 R	AATGCTYCCTGTGGGATTGAG	8275-8255		
		HPIV-3 Probe	(FAM) TCCCCATGGACATTCATTGTTTCCTGGTCT (BHQ-1)	8203-8174		
	HPIV-4	HPIV-4 AB F	CAAAYGATCCACAGCAAAGATTC	225-247	AB543336	(23)
	(Nucleoprotein)	HPIV-4 AB R	ATGTGGCCTGTAAGGAAAGCA	347-327		Modified
		HPIV-4 AB Probe	(VIC) GTATCATCATCTGCCAAAT (MGB)	278-296		
	HPIV-1	HPIV-1 F	CCATCCTTTTTCTGCAATGTATCC	7844-7867	NC_003461	Designed
	(HN)	HPIV-1 R	ATTGCAAACACTCTGATTAACATTGG	8000-7975		
		HPIV-1 Probe	(Cy5) CGGTGGCTTAACAACTCCGCTCCAAGG (BHQ-3)	7919-7945		
3	HBoV1	HBoV 2693f	GTCAACACAGAGCTTCCAATCC	2693-2714	NC_007455	(21)
	(NP-1)	HBoV 2781r	TGAATTAGTACCATCTCTAGCAATGC	2781-2756		
		HBoV 2724-TP	(FAM) AGTGCCAGTAGAACCCACACCACCCT (BHQ-1)	2749-2724		
	FLUA (H1N1) 2009	NIID-swH1 TMPrimer-F1	AGAAAAGAATGTAACAGTAACACACTCTGT	143-172	JX403978	(25)
	(Hemagglutinin)	NIID-swH1 TMPrimer-R1	TGTTTCCACAATGTARGACCAT	329-308		
		NIID-swH1 Probe1	(VIC) CAATRTTRCATTTACC (MGB)	255-240		
	HPIV-2	HPIV-2 F	GGACGCCTAAATATGGACCTCTC	7244-2266	NC_003443	Designed
	(HN)	HPIV-2 R	GTGAGTGTAACACCAATGGGTCT	7365-7343		
		HPIV-2 Probe	(Cy5) CCCAGCTTTATCCCCTCAGCAACATCTCCC (BHQ-3)	7276-7305		
4	FLUAV	MP-39-67For	CCMAGGTCGAAACGTAYGTTCTCTCTATC	39-67	JX163259	(25)
	(Matrix)	MP-183-153Rev	TGACAGRATYGGTCTTGTCTTTAGCCAYTCCA	184-153		
		MP-96-75ProbeAs	(FAM) ATYTCGGCTTTGAGGGGGCCTG (MGB)	96-75		
	FLUBV	Flu B F	TGGGAACAACAGCAACAAAAAA	257-278	AB120274	(18)
	(Matrix)	Flu B R	GCTGAGCTTTCATGGCCTTCT	368-348		
		Flu B Probe	(VIC) CATGAAGCATTTGAAATAG (MGB)	328-346		
	FLUCV	Flu C F	GACGACTACACACCAGACATCC	401-422	AB000721	Designed
	(Matrix)	Flu C R	CTGAGACATTACTCCTGTATCTTTCAC	494-468		
		Flu C Probe	(Cy5) TTGCATCTCAACCAAGCTGTGATTGTTCCT (BHQ-3)	456-427		
5	HCoV-NL63	HCoV-NL63 F	CTCTTTCTCAACCCAGGGCTG	26788-26808	NC_005831	Designed
	(Nucleocapsid)	HCoV-NL63 R	CGAGGACCAAAGCACTGAATAAC	26890-26868		
		HCoV-NL63 Probe	(FAM) ACCTCGTTGGAAGCGTGTTCCTACCA (BHQ-1)	26831-26856		
	HRV/HEV	Panenterhino/Ge/08 F2	CYAGCCTGCGTGG	364-376	EF173422	(22)
	(5′ UTR)	Panenterhino/Ge/08 F1	AGCCTGCGTGGCKGCC	366-381		Modified
		Entero/Panenterhino/Ge/08 R	GAAACACGGACACCCAAAGTAGT	569-547		
		Panenterhino/Ge/08-probe	(VIC) CCCTGAATGYGGCTAA (MGB)	459-474		
	HCoV-OC43	HCoV-OC43 F	GGGTACTGGTACAGACACAACAG	29398-29420	NC_005147	Designed
	(Nucleocapsid)	HCoV-OC43 R	GGTGCCGTACTGGTCTTTAGC	29523-29503		
		HCoV-OC43 Probe	(Cy5) CCGATGGCAACCAGCGTCAACTGCT (BHQ-3)	29438-29462		
6	HCoV-HKU1	HCoV-HKU1 F	CCTTGCGAATGAATGTGCTCAA	15525-15546	NC_006577	Designed
	(orf1ab)	HCoV-HKU1 R	GAGAACAAACATTAGCAGTAACAGC	15682-15658		
		HCoV-HKU1 Probe	(FAM) ACCACCAGGCTTAACATAATAGCAACCGCC (BHQ-1)	15600-15571		
	HEV	Entero/Ge/08 F	GCTGCGYTGGCGGCC	359-373	JQ965759	(22)
	(5′ UTR)	Entero/Panenterhino/Ge/08 R	GAAACACGGACACCCAAAGTAGT	564-542		Modified
		Panenterhino/Ge/08 Probe	(VIC) CCCTGAATGYGGCTAA (MGB)	454-469		
	HCoV (229E)	HCoV-229E F	GAGTCAGGCAACACTGTGGTC	26619-26639	NC_002645	Designed
	(Nucleocapsid)	HCoV-229E R	TGTTGTTGCATTTCTCTAGTGAATG	26737-26713		
		HCoV-229E Probe	(Cy5) AGAGTGACTGTGCCCAAAGACCATCCAC (BHQ-3)	26655-26682		
7	HAdV	Ad2-F	CCAGGACGCCTCGGAGTA	18888-18905	AC_000007	(24)
	(Hexon)	Ad2-R	AAACTTGTTATTCAGGCTGAAGTACGT	18972-18946		Modified
		Ad2-probe	(FAM) AGTTTGCCCGCGCCACCG (BHQ-1)	18926-18943		
	HAdV	Ad4-F	GGACAGGACGCTTCGGAGTA	18307-18326	AY458656	(24)
	(Hexon)	Ad4-R	CTTGTTCCCCAGACTGAAGTAGGT	18390-18367		Modified
		Ad4-probe	(Cy5) CAGTTCGCCCGYGCMACAG (BHQ-3)	18346-18364		

HAdV, human adenovirus; HBoV 1, human bocavirus 1; HCoV, human coronavirus; HEV, human enterovirus; HPIV, human parainfluenza virus; hMPV, human metapneumovirus; HRV, human rhinovirus; FLUAV, influenza virus A; FLUBV, influenza virus B; FLUCV, influenza virus C; RSV, respiratory syncytial virus; HN, hemagglutinin-neuraminidase; UTR, untranslated region.

Table 2. Monthly distribution of respiratory viruses detected between January 2010–December 2011

Virus	2010 Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	2011 Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total/Single/Multiple detections	Proportion (%)[1]
HAdV	2 (2)[2]	2 (1)	4 (3)	7 (7)	10 (8)	6 (4)	6 (6)	2 (0)	17 (16)	9 (8)	5 (2)	14 (10)	5 (3)	8 (7)	2 (1)	9 (7)	10 (9)	6 (6)	7 (3)	9 (8)	8 (6)	4 (3)	3 (3)	3 (2)	158/33/125	79.1
HBoV 1	1 (1)	1 (1)	3 (3)	20 (17)	13 (11)	7 (6)	9 (9)	—	8 (5)	4 (3)	2 (2)	2 (2)	5 (4)	7 (6)	4 (4)	27 (25)	33 (28)	15 (13)	4 (4)	4 (2)	3 (2)	2 (2)	—	2 (2)	176/24/152	86.4
HCoV-NL63	—	—	1 (1)	1 (1)	—	—	2 (2)	—	6 (3)	2 (2)	1 (1)	2 (2)	5 (4)	1 (1)	—	—	—	—	—	—	—	—	—	1 (1)	22/4/18	81.8
HCoV-229E	—	—	1 (1)	—	—	1 (1)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2/0/2	100
HCoV-OC43	—	—	1 (1)	1 (0)	—	5 (4)	4 (3)	1 (0)	1 (1)	2 (0)	1 (1)	3 (1)	5 (3)	7 (3)	3 (2)	4 (3)	—	—	1 (1)	—	1 (1)	—	—	—	40/16/24	60.0
HCoV-HKU1	—	1 (1)	1 (1)	—	—	—	—	—	—	—	—	—	—	—	—	2 (2)	1 (1)	—	—	1 (0)	—	—	—	—	6/1/5	83.3
HEV	1 (1)	—	—	2 (2)	2 (2)	2 (1)	9 (5)	7 (3)	5 (3)	2 (2)	1 (1)	1 (1)	—	—	—	1 (0)	2 (2)	2 (1)	3 (3)	4 (3)	6 (5)	2 (1)	—	—	52/16/36	69.2
HPIV-1	—	—	—	2 (1)	1 (0)	1 (0)	3 (1)	—	1 (0)	2 (2)	—	—	—	—	1 (0)	5 (0)	1 (0)	2 (1)	3 (0)	7 (2)	4 (2)	3 (2)	—	1 (0)	37/26/11	29.7
HPIV-2	—	—	—	—	—	—	—	1 (1)	3 (0)	—	2 (1)	3 (2)	—	—	—	—	—	—	—	1 (0)	—	—	—	1 (1)	11/6/5	45.5
HPIV-3	1 (1)	—	1 (1)	17 (12)	15 (13)	4 (1)	3 (3)	—	1 (1)	—	—	—	—	2 (2)	—	7 (6)	25 (19)	21 (11)	9 (6)	4 (1)	3 (2)	—	1 (1)	—	114/34/80	70.2
HPIV-4	—	—	—	—	—	1 (1)	2 (2)	2 (0)	3 (3)	4 (2)	1 (1)	4 (4)	2 (1)	5 (5)	—	1 (0)	1 (0)	—	1 (1)	3 (2)	4 (2)	5 (0)	1 (1)	3 (1)	43/17/26	60.5
hMPV	1 (0)	2 (2)	12 (4)	6 (5)	2 (1)	—	—	—	—	—	—	—	2 (1)	1 (1)	12 (4)	28 (19)	10 (7)	7 (3)	6 (2)	3 (1)	1 (1)	1 (0)	—	—	94/43/51	54.3
HRV	4 (2)	1 (1)	7 (4)	20 (16)	16 (13)	16 (8)	13 (9)	5 (1)	12 (7)	12 (8)	23 (7)	16 (12)	12 (10)	12 (10)	13 (5)	34 (23)	32 (26)	31 (21)	19 (12)	27 (12)	19 (12)	24 (7)	13 (5)	9 (6)	390/153/237	60.8
FLUAV[3]	—	1 (0)	—	—	—	1 (1)	—	—	—	—	—	—	4 (2)	3 (0)	2 (1)	1 (1)	—	—	—	—	—	—	2 (0)	—	14/7/7	50.0
FLUA (H1N1) 2009	9 (3)	5 (0)	2 (1)	—	—	—	—	—	—	—	—	7 (2)	13 (4)	5 (0)	—	—	—	—	—	—	—	—	—	2 (1)	43/30/13	30.2
FLUBV	—	—	—	—	—	—	—	—	—	1 (0)	1 (0)	—	1 (0)	1 (0)	1 (0)	3 (1)	1 (1)	—	—	—	—	—	—	—	9/7/2	22.2
FLUCV	1 (1)	1 (1)	1 (1)	—	—	1 (1)	—	—	—	—	—	—	—	1 (0)	—	—	—	—	—	—	—	—	—	—	5/1/4	80.0
RSV A	6 (3)	2 (1)	5 (2)	2 (2)	1 (1)	4 (3)	9 (6)	4 (1)	10 (7)	—	6 (2)	6 (3)	5 (4)	1 (0)	3 (1)	1 (1)	2 (0)	4 (3)	17 (9)	11 (5)	5 (5)	5 (2)	4 (2)	5 (2)	118/52/66	55.9
RSV B	—	—	—	2 (2)	—	—	2 (1)	—	11 (8)	10 (8)	5 (3)	12 (7)	12 (5)	1 (0)	4 (2)	4 (3)	1 (1)	3 (0)	5 (1)	3 (3)	2 (0)	1 (1)	—	2 (2)	80/33/47	58.8
Total detection	26 (14)	16 (8)	39 (23)	80 (65)	60 (49)	49 (31)	62 (47)	22 (6)	78 (54)	48 (35)	48 (21)	70 (46)	71 (41)	55 (40)	45 (20)	127 (91)	119 (94)	91 (59)	75 (42)	77 (39)	56 (38)	47 (18)	24 (12)	29 (18)	1,414/503/911	
Virus-positive specimens (Multiple-virus positives)	18 (6)	12 (4)	26 (10)	41 (26)	29 (18)	31 (13)	34 (19)	19 (3)	45 (21)	28 (15)	37 (10)	44 (20)	50 (21)	32 (17)	35 (10)	72 (36)	64 (39)	58 (26)	51 (18)	55 (16)	36 (18)	37 (8)	18 (6)	19 (8)	891 (388)[4]	43.5
No. tested specimens	19	14	28	44	32	34	39	27	52	33	43	48	61	38	39	76	69	63	63	65	51	48	32	26	1,044[5]	
Virus positivity rate in specimens (%)	94.7	85.7	92.9	93.2	90.6	91.2	87.2	70.4	86.5	84.8	86.0	91.7	82.0	84.2	89.7	94.7	92.8	92.1	81.0	84.6	70.6	77.1	56.3	73.1	85.3	

[1]: Proportion: Multiple virus-positive specimens/total virus-positive specimens.
[2]: Number of virus-positive specimens (number of multiple virus-positive specimens).
[3]: FLUAV: Numbers were counted as number of FLUAV-positive detections minus number of FLUA (H1N1) 2009-positive detections.
[4]: Total number of virus-positive specimens (total number of multiple virus-positive specimens).
[5]: Total number of specimens tested.
—, not detected.

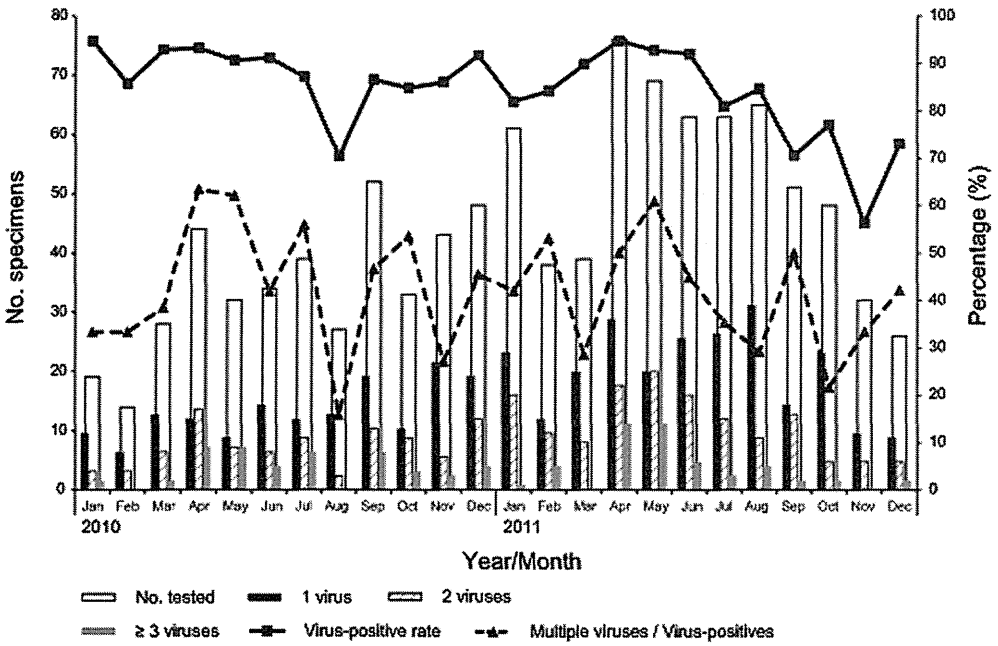

Fig. 1. Monthly distribution of respiratory virus infections in ARI children (<6 years of age) between January 2010–December 2011. The unbroken line with closed squares and the broken line with closed triangles represent percentages of virus-positive rates and the proportion of virus-positive specimens with multiple viruses, respectively. Numbers of specimens are shown as open bars. Cases of mono, dual, and ≥triple virus co-detections are shown as closed, hatched, and shaded bars, respectively.

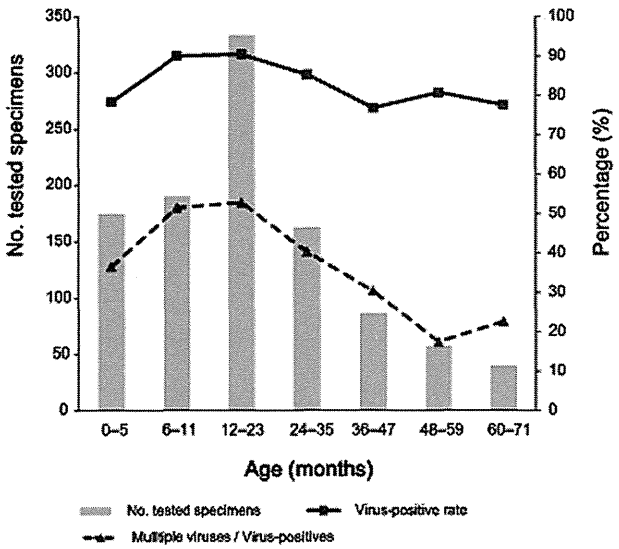

Fig. 2. Age-group stratified rates of multiple respiratory virus infections. Age groups were 0–5, 6–11, 12–23, 24–35, 36–47, 48–59, and 60–71 months. Numbers of specimens tested are shown as shaded bars. The unbroken line with closed squares and the broken line with closed triangles represent percentages of virus-positive rates and the proportion of virus-positive specimens with multiple viruses, respectively.

clusively co-detected.

DISCUSSION

In this study, multiple virus genes were detected in single specimens from children (<6 years), and a high ratio of multiple/total virus-positive specimens was observed in young children (<3 years).

In statistical analyses of pairs of co-detected viruses, we also obtained evidence that some combinations of viruses are synchronously or exclusively co-detected.

The combination of HBoV 1 and HAdV was demonstrated to be synchronously co-detected in the present study. Frequent co-detection of HBoV with HAdV was also reported previously (12). A high rate of co-detection of HBoV with HAdV was reported in hospitalized Israeli children and was not dependent on the HAdV serotype (29). Prolonged shedding of the HBoV 1 virus may partially contribute to the high rate of co-detection with other respiratory viruses (30). To our knowledge, this is the second report of synchronous co-detection of HBoV 1 with HAdV by statistical analysis (12). Our results also suggested that the increased frequency of co-detection does not overlap with the respective viral epidemic seasons, indicating a more complex causation mechanism. Tanner et al. suggested that co-detections are not random and that most associations are either positively or negatively reciprocal (13). It would be worthwhile to investigate whether HAdV affects HBoV replication or function or vice versa.

Exclusive co-detection of some combinations of viruses was observed in the present study. Most combinations were between viruses of the family *Paramyxoviridae*. This is consistent with a previous report of negatively correlated single-stranded RNA virus infections (10). In addition, exclusive co-detection of RSV A and RSV B was also suggested in a previous study (10). Greer et al. reported that RSV, hMPV, and HPIV infections did not occur together (11). In contrast, no statistically significant associations were reported between RSV, hMPV, and HPIV (13).

Considering that only a few studies have analyzed the

Table 3. Number of co-detections with each respiratory virus between January 2010–December 2011

	HAdV	HBoV 1	HCoV NL63	HCoV 229E	HCoV OC43	HCoV HKU1	HEV	HPIV-1	HPIV-2	HPIV-3	HPIV-4	hMPV	HRV	FLUAV	FLUA (H1N1) 2009	FLUBV	FLUCV	RSV A	RSV B
HAdV		6	1	—	—	1	2	1	—	3	—	5	28	—	3	—	—	5	10
HBoV 1	35		—	—	2	1	2	2	1	15	1	8	28	1	1	1	—	8	4
HCoV-NL63	4	1		—	—	—	1	—	—	1	1	2	5	—	—	—	—	1	1
HCoV-229E	—	—	—		—	—	—	—	—	—	—	1	—	—	—	—	—	—	—
HCoV-OC43	3	6	—	—		—	1	—	—	—	1	3	4	1	1	—	—	2	—
HCoV-HKU1	—	1	—	—	—		—	—	—	1	—	—	1	—	—	—	—	—	—
HEV	6	5	—	1	2	—		—	1	3	2	1	6	—	—	—	—	3	—
HPIV-1	—	1	—	—	—	—	—		—	—	—	—	7	—	—	—	—	—	—
HPIV-2	1	—	—	—	—	—	—	—		—	—	—	2	—	—	—	—	—	—
HPIV-3	16	31	—	—	1	—	3	—	—		1	1	18	—	1	—	—	1	—
HPIV-4	5	3	2	—	—	—	1	—	—	—		1	6	—	1	—	—	1	2
hMPV	6	10	—	—	—	1	—	—	—	1	—		14	—	—	1	1	—	—
HRV	43	55	2	—	5	1	9	1	1	28	6	12		1	1	—	—	21	12
FLUAV[1)]	1	2	—	—	—	1	—	—	—	—	—	1	2		—	—	—	—	1
FLUA (H1N1) 2009	1	2	—	—	—	—	—	—	—	—	—	—	2	—		—	—	1	2
FLUBV	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		—	—	—
FLUCV	—	1	—	1	—	—	1	—	—	—	—	—	1	—	—	—		—	—
RSV A	12	9	1	—	1	—	3	—	—	4	1	—	15	1	—	—	1		—
RSV B	11	6	2	—	2	—	3	—	—	1	2	—	8	—	—	—	—	—	

Upper right: Number of dual co-detections with each respiratory virus.
Lower left: Number of triple or more co-detections with each respiratory virus.
1): FLUAV: Values were counted as number of FLUAV-positive detections minus number of FLUA (H1N1) 2009-positive detections.
—, not detected.
Abbreviations are in Table 1.

pairs of co-detected respiratory viruses and no study had reported their associations in vitro or in vivo, the significance of the specific combinations of co-detected viruses is uncertain (10–13). In addition, this study was very limited because the specimens were collected from patients in only 1 city during a 2-year virus surveillance program. Therefore, further studies are required to confirm the associations between co-detected respiratory viruses and to demonstrate the mechanism underlying the associations that may lead to cooperation or competition between these pairs of viruses.

Acknowledgments The authors are grateful to all the pediatricians who collected specimens for this study.

This work was supported in part by a Grant-in-Aid for Young Scientists (B) (23790720) from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by a Grant-in-Aid for Emerging and Re-emerging Infectious Diseases (H23-Shinko-Japan-004) from the Ministry of Health, Labour and Welfare of Japan.

Conflict of interest None to declare.

REFERENCES

1. Eccles R. Understanding the symptoms of the common cold and influenza. Lancet Infect Dis. 2005;5:718-25.
2. Heikkinen T, Jarvinen A. The common cold. Lancet. 2003;361: 51-9.
3. Ren L, Gonzalez R, Wang Z, et al. Prevalence of human respiratory viruses in adults with acute respiratory tract infections in Beijing, 2005–2007. Clin Microbiol Infect. 2009;15:1146-53.
4. Stempel HE, Martin ET, Kuypers J, et al. Multiple viral respiratory pathogens in children with bronchiolitis. Acta Paediatr. 2009;98:123-6.
5. Zhang G, Hu Y, Wang H, et al. High incidence of multiple viral infections identified in upper respiratory tract infected children under three years of age in Shanghai, China. PLoS One. 2012;7: e44568.
6. da Silva ER, Pitrez MC, Arruda E, et al. Severe lower respiratory tract infection in infants and toddlers from a non-affluent population: viral etiology and co-detection as risk factors. BMC Infect Dis. 2013;13:41.
7. Gagliardi TB, Paula FE, Iwamoto MA, et al. Concurrent detection of other respiratory viruses in children shedding viable human respiratory syncytial virus. J Med Virol. 2013;85:1852-9.
8. Harada Y, Kinoshita F, Yoshida LM, et al. Does respiratory virus coinfection increases the clinical severity of acute respiratory infection among children infected with respiratory syncytial virus? Pediatr Infect Dis J. 2013;32:441-5.
9. Yoshida LM, Suzuki M, Nguyen HA, et al. Respiratory syncytial virus: co-infection and paediatric lower respiratory tract infections. Eur Respir J. 2013;42:461-9.
10. Brunstein JD, Cline CL, McKinney S, et al. Evidence from multiplex molecular assays for complex multipathogen interactions in acute respiratory infections. J Clin Microbiol. 2008;46:97-102.
11. Greer RM, McErlean P, Arden KE, et al. Do rhinoviruses reduce the probability of viral co-detection during acute respiratory tract infections? J Clin Virol. 2009;45:10-5.
12. Martin ET, Fairchok MP, Stednick ZJ, et al. Epidemiology of multiple respiratory viruses in childcare attendees. J Infect Dis. 2013;207:982-9.
13. Tanner H, Boxall E, Osman H. Respiratory viral infections during the 2009–2010 winter season in Central England, UK: incidence and patterns of multiple virus co-infections. Eur J Clin Microbiol Infect Dis. 2012;31:3001-6.
14. Allander T, Tammi MT, Eriksson M, et al. Cloning of a human parvovirus by molecular screening of respiratory tract samples. Proc Natl Acad Sci USA. 2005;102:12891-6.
15. Woo PC, Lau SK, Chu CM, et al. Characterization and complete genome sequence of a novel coronavirus, coronavirus HKU1, from patients with pneumonia. J Virol. 2005;79:884-95.
16. Liao RS, Tomalty LL, Majury A, et al. Comparison of viral isolation and multiplex real-time reverse transcription-PCR for confirmation of respiratory syncytial virus and influenza virus detection by antigen immunoassays. J Clin Microbiol. 2009;47:527-32.
17. Mahony JB. Detection of respiratory viruses by molecular methods. Clin Microbiol Rev. 2008;21:716-47.
18. Kaida A, Kubo H, Takakura K, et al. Molecular epidemiology of human rhinovirus C in patients with acute respiratory tract infections in Osaka City, Japan. Jpn J Infect Dis. 2011;64:488-92.
19. Neske F, Blessing K, Tollmann F, et al. Real-time PCR for diagnosis of human bocavirus infections and phylogenetic analysis. J Clin Microbiol. 2007;45:2116-22.
20. Kaida A, Kubo H, Shiomi M, et al. Evaluation of real-time RT-PCR compared with conventional RT-PCR for detecting human metapneumovirus RNA from clinical specimens. Jpn J Infect Dis. 2008;61:461-4.
21. Kaida A, Kubo H, Takakura K, et al. Detection and quantitative analysis of human bocavirus associated with respiratory tract infection in Osaka City, Japan. Microbiol Immunol. 2010;54: 276-81.
22. Tapparel C, Cordey S, Van Belle S, et al. New molecular detection tools adapted to emerging rhinoviruses and enteroviruses. J Clin Microbiol. 2009;47:1742-9.
23. van de Pol AC, van Loon AM, Wolfs TF, et al. Increased detection of respiratory syncytial virus, influenza viruses, parainfluenza viruses, and adenoviruses with real-time PCR in samples from patients with respiratory symptoms. J Clin Microbiol. 2007;45: 2260-2.
24. Wong S, Pabbaraju K, Pang XL, et al. Detection of a broad range of human adenoviruses in respiratory tract samples using a sensitive multiplex real-time PCR assay. J Med Virol. 2008;80: 856-65.
25. World Health Organization (WHO). WHO information for laboratory diagnosis of pandemic (H1N1) 2009 virus in humans-revised. 2009. Available at <http://www.who.int/csr/resources/publications/swineflu/diagnostic _ recommendations/en/index.html>.
26. Kaida A, Kubo H, Sekiguchi J, et al. Enterovirus 68 in children with acute respiratory tract infections, Osaka, Japan. Emerg Infect Dis. 2011;17:1494-7.
27. Edgington ES, Onghena P. Randomization tests. Statistics, textbooks and monographs. Boca Raton, FL: Chapman & Hall/CRC; 2007.
28. R Core Team. R: A Language and Environment for Statistical Computing. Vienna, Austria; 2012.
29. Hindiyeh MY, Keller N, Mandelboim M, et al. High rate of human bocavirus and adenovirus coinfection in hospitalized Israeli children. J Clin Microbiol. 2008;46:334-7.
30. Martin ET, Fairchok MP, Kuypers J, et al. Frequent and prolonged shedding of bocavirus in young children attending daycare. J Infect Dis. 2010;201:1625-32.