表2. 小児呼吸器由来臨床検体を用いたマルチプレックス・リアルタイム(RT-)PCR法における
採取月別病原ウイルス遺伝子検出数

遺伝子検出病原ウイルス	臨床検体採取月										合計
	平成 26.4	5	6	7	8	9	10	11	12	平成 27.1	
アデノウイルス	2	4	1	2	4	2	0	1	0	2	18
コロナウイルス NL63	—	—	—	—	1	—	—	—	—	—	1
コロナウイルス 229E	—	1	—	—	—	—	—	—	—	—	1
コロナウイルス OC43	—	1	1	1	—	1	2	2	2	1	11
コロナウイルス HKU1	—	—	—	—	—	—	—	—	—	—	—
エンテロウイルス	—	—	1	2	3	1	2	—	1	—	10
ヒトボカウイルス	3	7	5	—	—	—	—	—	—	1	16
ヒトメタニューモウイルス	5	5	1	—	—	—	1	—	—	5	17
インフルエンザウイルス A 型	—	—	—	—	—	—	—	—	—	3	3
インフルエンザウイルス AH1pdm09	—	—	—	—	—	—	—	—	—	—	—
インフルエンザウイルス B 型	1	—	—	—	—	—	—	—	—	—	1
インフルエンザウイルス C 型	—	—	—	—	—	—	—	—	—	—	—
パラインフルエンザウイルス 1 型	—	1	—	—	1	—	1	—	1	—	4
パラインフルエンザウイルス 2 型	—		—	3	4	3	1	—	—	—	11
パラインフルエンザウイルス 3 型	—	7	10	1	1	1	—	—	—	—	20
パラインフルエンザウイルス 4 型	—	1	—	—	—	1	1	3	—	1	7
ライノウイルス	7	14	10	7	3	8	10	9	4	3	75
RS ウイルス A	—	—	—	—	4	1	4	2	6	4	21
RS ウイルス B	—	—	—	1	2	5	1	2	—	1	12

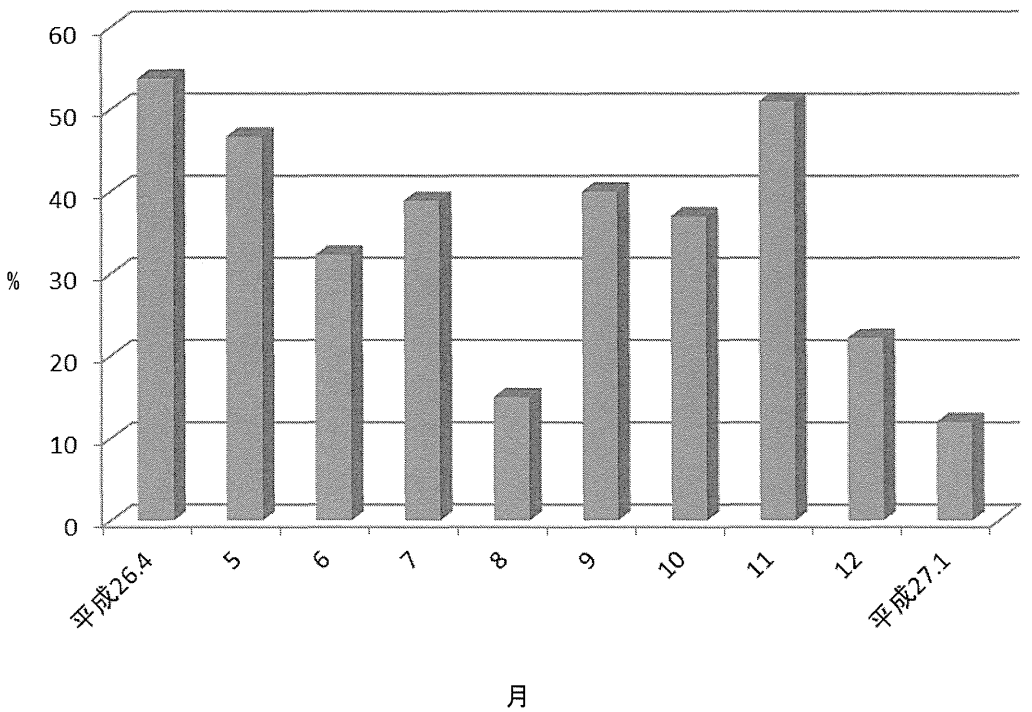

図1. 採取月別検体数におけるライノウイルス遺伝子検出陽性検体数の割合 (%)

Ⅲ. 学会等発表実績

様式第１９

学　会　等　発　表　実　績

委託業務題目「海外研究機関等との感染症に関する共同研究および連携強化に関する研究」
機関名 国立感染症研究所

1．学会等における口頭・ポスター発表

発表した成果（発表題目、口頭・ポスター発表の別）	発表者氏名	発表した場所（学会等名）	発表した時期	国内・外の別
ARDSから多臓器不全に至ったインフルンザA/H1pdm重症肺炎成人例におけるウイルス学的検討（口頭発表）	大場邦弘、高橋 仁、高山郁代、中内美名、影山 努	第28回インフルエンザ研究者交流の会シンポジウム、鳥取市	2014年7月	国内
The host protease TMPRSS2 is essential for influenza A virus pathogenicity（口頭発表）	Kouji Sakai, Yasushi Ami, Maino Tahara, Toru Kubota, Masaki Anraku, Noriko Nakajima, Tsuyoshi Sekizuka, Katsuhiro Komase, Makoto Kuroda, Hideki Hasegawa, Yoshihiro Kawaoka, Masato Tashiro, Makoto Takeda	13th Awaji International forum on infection and immunity in Nara	2014年9月	国内
宿主プロテアーゼTMPRSS2は、インフルエンザウイルスの生体内活性化酵素である（口頭発表）	酒井宏治、網 康至、田原舞乃、久保田耐、安楽正輝、中島典子、関塚剛史、駒瀬勝啓、長谷川秀樹、黒田 誠、河岡義裕、田代眞人、竹田 誠	第157回日本獣医学会学術集会、札幌市	2014年9月	国内
犬ジステンパーウイルスのヒトSLAM利用に必要な受容体側の因子（口頭発表）	酒井宏治、關 文緒、田原舞乃、網 康至、山口良二、駒瀬勝啓、竹田 誠	第157回日本獣医学会学術集会、札幌市	2014年9月	国内
宿主プロテアーゼTMPRSS2はセンダイウイルスの病原性決定因子のひとつである（口頭発表）	北沢実乃莉、酒井宏治、田原舞乃、安部昌子、中島勝紘、網v康至、中島典子、安楽正輝、駒瀬勝啓、長谷川秀樹、竹原一明、田代眞人、加藤v篤、竹田v誠	第157回日本獣医学会学術集会、札幌市	2014年9月	国内
麻しん診断例から検出された麻しんウイルス株の分子疫学解析（大阪市 2007～2014年）（口頭発表）	改田 厚、入谷展弘、山元誠司、平井有紀、廣川秀徹、影山v努、久保英幸	第46回日本小児感染症学会、東京	2014年10月	国内
Frequent respiratory viral infections in a young child in a 27-month follow-up study（ポスター発表）	Atsushi Kaida, Hideyuki Kubo, Nobuhiro Iritani, Seiji P. Yamamoto, Atsushi Hase, Tsutomu Kageyema	International Congress on Medical Virology 2014 in Thailand, Bangkok	2014年11月	国外
大阪市の麻しん、および発しん性ウイルス感染症の疫学的検査とその考察（口頭発表）	廣川秀徹、改田 厚、久保英幸、山元誠司、入谷展弘、坂本徳裕、松本珠実、伯井紀隆、安井典子、桝田晴美、藤森良子、細井舞子、大平真由、奥町彰礼、半羽宏之、西尾孝之、吉田英樹、松本健二、竹内 敏、甲田伸一、吉村高尚	第38回大阪府医師会医学会総会、大阪市	2014年11月	国内
2014年にベトナムでヒト感染が確認された高病原性鳥インフルエンザA(H5N1)ウイルスの遺伝子解析（ポスター発表）	高山郁代、Nguyen Trung Hieu、中内美名、高橋 仁、Nguyen Thanh Long、小田切孝人、田代眞人、影山 努	第62回日本ウイルス学会学術集会、横浜市	2014年11月	国内
RT-LAMP法を用いたA/H7N9亜型鳥インフルエンザウイルス検出系の構築（ポスター発表）	中内美名、高山郁代、大場邦弘、高橋仁、田代眞人、影山 努	第62回日本ウイルス学会学術集会、横浜市	2014年11月	国内
野生型イヌジステンパーウイルスのヒトSLAM利用能獲得に必要な変異（口頭発表）	酒井宏治、關 文緒、加納和彦、網 康至、田原舞乃、駒瀬勝啓、前仲勝実、山口良二、竹田 誠	第62回日本ウイルス学会学術集会、横浜市	2014年11月	国内
中東呼吸器症候群コロナウイルス（MERS-CoV）の RT-LAMP 法による検査法の開発（ポスター発表）	白戸憲也、矢野拓弥、仙波晶平、赤地重宏、小林隆司、西中隆道、納富継宣、松山州徳	第62回日本ウイルス学会学術集会、横浜市	2014年11月	国内

2013/14シーズンに分離されたインフルエンザウイルスAH3亜型株の性状解析－大阪市(ポスター発表)	久保英幸、改田 厚、山元誠司、入谷展弘	第62回日本ウイルス学会学術集会、横浜市	2014年11月	国内
Comparative analysis for the detection of avian influenza H5N1 virus by using a novel luminescence analyzer(POCube) and real-time RT-PCR.	A Yoppy R Candra, Anna L Poetranto, Aldise M Nastri, Edith F Puruhito, 横田(恒次)恭子, 西村研吾, 影山 努, 高原悠佑, 堀田 博, 清水一史.	第62回日本ウイルス学会学術集会. 横浜	2014年11月	国内
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麻疹・風疹に関する最近の国内疫学情報について(口頭発表)	多屋馨子、佐藤弘、奥野英雄、新井智、神谷 元、八幡裕一郎、伊東宏明、福住宗久、砂川富正、駒瀬勝啓、竹田誠、大石和徳	第18回日本ワクチン学会学術集会、福岡市	2014年12月	国内

2. 学会誌・雑誌等における論文掲載

掲載した論文(発表題目)	発表者氏名	発表した場所(学会誌・雑誌等名)	発表した時期	国内・外の別
海外の麻疹の情報 2013	駒瀬勝啓、竹田 誠	病原微生物検出情報	2014年4月	国内
潜在的な疫学リンクが疑われたD8型ウイルスによる麻疹広域散発事例	山岸拓也、伊東宏明、八幡裕一郎、中島一敏、松井珠乃、高橋琢理、木下一美、砂川富正、奥野英雄、多屋馨子、大石和徳、駒瀬勝啓、三崎貴子、丸山絢、大嶋孝弘、清水英明、岩瀬耕一、岡部信彦、小泉祐子、平岡麻理子、瀬戸成子、杉本徳子、荷見奈緒美、熊谷行広、大塚吾郎、杉下由行、甲賀健史、鈴木理恵子、阿南弥生子、舟久保麻理子、弘光明子、坂本洋、阿部勇治、氏家無限	病原微生物検出情報	2014年4月	国内
Real-time RT-PCR assays for discriminating influenza B virus Yamagata and Victoria lineages.	Mina Nakauchi, Ikuyo Takayama, Hitoshi Takahashi, Kunihiro Oba, Hideyuki Kubo, Atsushi Kaida, Masato Tashiro, Tsutomu Kageyama.	Journal of Virological Methods	2014年5月	国外
フィリピン渡航者～のD9型麻しんルイスの検出-福岡市	古川英臣、梶山桂子、宮代 守、佐藤正雄、伊藤孝子、酒井由美子、井出瑤子、植山 誠、眞野理恵子、衣笠有紀、戸川 温、高田 徹、猪狩洋介、駒瀬勝啓	病原微生物検出情報	2014年5月	国内
Ongoing increase in measles cases following importations, Japan, Marchi 2014: times of challenge and opportunity.	Takuri Takahashi, Yuzo Arima, Hitomi Kinoshita, Kazuhiko Kanou, Takehito Saitoh, Tomimasa Sunagawa, Hiroaki Ito, Atsuhiro Kanayama, Ayako Tabuchi, Kazutoshi Nakashima, Yuichiro Yahata, Takuya Yamagishi, Tamie Sugawara, Yasushi Ohkusa, Tamano Matsui, Satoru Arai, Hiroshi Satoh, Keiko Tanaka-Taya, Katsuhiro Komase, Makoto Takeda, Kazunori Oishi.	Western Pac Surveill Rresponse Journals	2014年5月	国外
The host protease TMPRSS2 plays a major role in in vivo replication of emerging H7N9 and seasonal influenza viruses	Kouji Sakai, Yasushi Ami, Maino Tahara, Toru Kubota, Masaki Anraku, Masako Abe, Noriko Nakajima, Tsuyoshi Sekizuka, Kazuya Shirato, Yuriko Suzaki, Akira Ainai, Yuichiro Nakatsu, Kazuhiko Kanou, Kazuya Nakamura, Tadaki Suzuki, Katsuhiro Komase, Eri Nobusawa, Katsumi Maenaka, Makoto Kuroda, Hideki Hasegawa, Yoshihiro Kawaoka, Masato Tashiro, Makoto Takeda	Journal of Virology	2014年5月	国外
Broad cross-reactive epitopes of the H5N1 influenza virus identified by murine antibodies against the A/Vietnam/1194/2004 hemagglutinin.	Mie Kobayashi-Ishihara, Hitoshi Takahashi, Kazuo Ohnishi, Kengo Nishimura, Kazutaka Terahara, Manabu Ato, Sigeyuki Itamura, Tsutomu Kageyama, Yasuko Tsunetsugu-Yokota.	PLoS One	2014年6月	国外
輸入麻疹と国内伝播	竹田 誠、駒瀬勝啓	感染症	2014年6月	国内
Development of a sensitive novel diagnostic kit for the highly pathogenic avian influenza A (H5N1) virus.	Yasuko Tsunetsugu-Yokota, Kengo Nishimura, Shuhei Misawa, Mie Kobayashi-Ishihara, Hitoshi Takahashi, Ikuyo Takayama, Kazuo Ohnishi, Shigeyuki Itamura, Hang L. K. Nguyen, Mai T. Q. Le, Giang T. Dang, Long T. Nguyen, Masato Tashiro, Tsutomu Kageyama.	BMC Infectious Diseases	2014年7月	国外

Development of a reverse transcription loop-mediated isothermal amplification assay for the rapid diagnosis of avian influenza A (H7N9) virus infection.	Mina Nakauchi, Ikuyo Takayama, Hitoshi Takahashi, Masato Tashiro, Tsutomu Kageyama.	Journal of Virological Methods	2014年8月	国外
Detection of Middle East respiratory syndrome coronavirus using reverse transcription loop-mediated isothermal amplification (RT-LAMP).	Kazuya Shirato, Takuya Yano, Syouhei Senba, Shigehiro Akachi, Takashi Kobayashi, Takamichi Nishinaka, Tsugunori Notomi, and Shutoku Matsuyama	Virology Journal	2014年8月	国外
Development of an improved RT-LAMP assay for detection of currently circulating rubella viruses.	Abo H, Okamoto K, Anraku M, Otsuki N, Sakata M, Icenogle J, Zheng Q, Kurata T, Kase T, Komase K, Takeda M, Mori Y.	Journal of Virological Methods	2014年10月	国外
Frequent respiratory viral infections in a young child in a 27-month follow-up study	Atsushi Kaida, Hideyuki Kubo, Nobuhiro Iritani, Seiji P. Yamamoto, Atsushi Hase, Koh-ichi Takakura, Tsutomu Kageyema	JMM Case Reports	2014年10月	国外
Global update on the susceptibility of human influenza viruses to neuraminidase inhibitors, 2012-2013	Adam Meijer, Helena Rebelo-de-Andrade, Vanessa Correia, Terry Besselaar, Renu Drager Dayal, Alicia Fry, Vicky Gregory, Larisa Gubareva, Tsutomu Kageyama, Angie Lackenby, Janice Lo, Takato Odagiri, Dmitriy Pereyaslov, Marilda M. Siqueira, Emi Takashita, Masato Tashiro, Dayan Wang, Sun Wong, Wenqing Zhang, Rod S. Daniels, Aeron C. Hurt.	Antiviral Research.	2014年10月	国外
Epitope Mapping of the Hemagglutinin Molecule of A/(H1N1)pdm09 Influenza Virus by Using Monoclonal Antibody Escape Mutants	Yoko Matsuzaki, Kanetsu Sugawara, Mina Nakauchi, Yoshimasa Takahashi, Taishi Onodera, Yasuko Tsunetsugu-Yokota, Takayuki Matsumura, Manabu Ato, Kazuo Kobayashi, Yoshitaka Shimotai, Katsumi Mizuta, Seiji Hongo, Masato Tashiro, Eri Nobusawa	Journal of Virology	2014年8月	国外
インフルエンザA/H1 pdm09亜型及びA/H3亜型感染症による小児の入院診断名の比較	大場邦弘、加藤昭生、古谷智子、小花奈都子、林健太、村田岳哉、野田雅裕、石川涼子、吉田知広、野田絵理、小鍛治雅之、高橋 仁、高山郁代、中内美名、影山 努	小児科臨床	2014年12月	国内
Associations between co-detected respiratory viruses in children with acute respiratory infections	Atsushi Kaida, Hideyuki Kubo, Koh-ichi Takakura, Jun-ichiro Sekiguchi, Seiji P. Yamamoto, Urara Kohdera, Masao Togawa, Kiyoko Amo, Masashi Shiomi, Minori Ohyama, Kaoru Goto, Atsushi Hase, Tsutomu Kageyama, Nobuhiro Iritani	Japanese Journal of Infectous Diseases	2014年12月	国内
要注意！ インフルエンザの季節到来！	久保英幸	健康・環境・サイエンス	2014年12月	国内
2013/14シーズンに大阪市内で分離された季節性インフルエンザウイルス	久保英幸、改田厚、入谷展弘、山元誠司、長谷篤、西尾孝之	大阪市立環科研報告	2014年12月	国内
Development of a diagnostic system for novel influenza A(H7N9) virus using real-time RT-PCR assay in Japan.	Ikuyo Takayama, Hitoshi Takahashi, Mina Nakauchi, Shiho Nagata, Masato Tashiro, Tsutomu Kageyama.	Japanese Journal of Infectous Diseases	in press	国内

Ⅳ. 研究成果の刊行物・別刷

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Short communication

Development of a reverse transcription loop-mediated isothermal amplification assay for the rapid diagnosis of avian influenza A (H7N9) virus infection

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ABSTRACT

A genetic diagnosis system for detecting avian influenza A (H7N9) virus infection using reverse transcription-loop-mediated isothermal amplification (RT-LAMP) technology was developed. The RT-LAMP assay showed no cross-reactivity with seasonal influenza A (H3N2 and H1N1pdm09) or influenza B viruses circulating in humans or with avian influenza A (H5N1) viruses. The sensitivity of the RT-LAMP assay was 42.47 copies/reaction. Considering the high specificity and sensitivity of the assay for detecting the avian influenza A (H7N9) virus and that the reaction was completed within 30 min, the RT-LAMP assay developed in this study is a promising rapid diagnostic tool for avian influenza A (H7N9) virus infection.

Many subtypes of avian influenza A viruses, including H7N2, H7N3, H9N2, H10N7, and H6N1, infect humans sporadically and cause respiratory illness (Arzey et al., 2012; Reperant et al., 2012; Shi et al., 2013). Cases of humans infected with highly pathogenic avian influenza A (H5N1) viruses have been reported from Asia to Europe and Africa, causing a total of 641 infections and 380 deaths as of October 8, 2013 (http://www.who.int/influenza/human_animal_interface/EN_GIP_20131008CumulativeNumberH5N1cases.pdf). In 2003 in the Netherlands, endemic highly pathogenic avian influenza A (H7N7) virus infections were reported in 90 cases, one of which was fatal (Fouchier et al., 2004; Koopmans et al., 2004).

The first human cases of infection with the avian influenza A (H7N9) virus (A/H7N9) were reported in late March 2013, and the virus was confirmed to have caused 137 infections and 45 deaths in mainland China as of October 25, 2013 (http://www.who.int/influenza/human_animal_interface/influenza_h7n9/10u_ReportWebH7N9Number.pdf) (Gao et al., 2013; Li et al., 2013a). More than 200 human cases have been reported in China within the first 2 months of 2014. Considering that probable human-to-human transmission of the A/H7N9 virus between 2 patients was reported recently and that the airborne transmissibility of the virus between ferrets (a mammalian model) has been reported by several groups, further spread of the infection is of increasing concern (Belser et al., 2013; Qi et al., 2013; Watanabe et al., 2013; Zhang et al., 2013b; Zhu et al., 2013).

A novel nucleic acid amplification method, loop-mediated isothermal amplification (LAMP), was reported in the early 2000s and the reverse transcription (RT)-LAMP assay can be performed without the need for high-precision instruments and can detect viral genomes within 30 min (Nagamine et al., 2002; Notomi et al., 2000). A/Shanghai/1/2013, A/Shanghai/2/2013, and A/Anhui/1/2013 were isolated from the first three human cases of A/H7N9 infection by using embryonated eggs at the Chinese Center for Disease Control and Prevention, and the sequences of the coding regions of all eight viral genes were deposited quickly in the influenza sequence database of the Global Initiative on Sharing All Influenza Data (GISAID) on March 31, 2013. On the basis of these sequence data, several RT-PCR and RT-LAMP methods targeting A/H7N9 viruses have been developed (Corman et al., 2013; Ge et al., 2013; Hackett et al., 2013; Li et al., 2013b; Nie et al., 2013; Wong et al., 2013; Zhang et al., 2013a) (http://www.who.int/influenza/gisrs_laboratory/cnic_realtime_rt_pcr_protocol_a_h7n9.pdf). Previously, the direct RT-LAMP method was established to detect viral RNA directly from nasal or nasopharyngeal swabs dispensed in an extraction reagent without an RNA purification step (Nakauchi et al., 2011b). In the present study, with the aim of providing a highly specific and sensitive diagnostic

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0166-0934/

Table 1
Primer set.

Primer name	Sequence (5′–3′)	Position[c]	Length (bp)
AH7N9-F3	TTCCTGAGATTCCAAA	995–1010	16
AH7N9-B3	GGTTGGTTTTTTCTATAAGCCG	1177–1198	22
AH7N9-FIP[a]	ACCAACCATCAATTAGGCCTT-	1061–1081 (F1c)	40
(F1c+F2)	CTATTTGGTGCTATAGCGG	1021–1039 (F2)	
AH7N9-BIP[b]	GGTTTCAGACACCAGAATGCACA-	1084–1106 (B1c)	45
(B1c+B2)	CCTGTTATTTGATCAATTGCCG	1145–1166 (B2)	
AH7N9-LF	CCCATCCATTTTCAATGAAAC	1040–1060	21
AH7N9-LB	ACTGCTGCAGATTACAAAAG	1117–1136	20

[a] AH7N9-FIP primer consisted of F1c and F2.
[b] AH7N9-BIP primer consisted of B1c and B2.
[c] The nucleotide positions of the HA gene of A/Anhui/1/2013 are based on the cRNA sequence obtained from the GISAID database (isolate ID number: EPI_ISL_138739).

Table 2
The specificity of the RT-LAMP assay was tested using serial dilutions of viral RNA from H7 subtype avian influenza A viruses.

Virus	Viral RNA concentrations (copies/μL)[a]	Number of positive replicates/number of tests for each assay				
		Dilution rate of viral RNA				
		10^1	10^2	10^3	10^4	10^5
A/Anhui/1/2013 (H7N9)	1.5×10^5	2/2	2/2	2/2	2/2	0/2
A/duck/Fukui/1/2004 (H7N7)	1.5×10^5	2/2	1/2	0/2	0/2	0/2
A/mallard/Netherlands/12/2000 (H7N3)	2.9×10^7	NT	NT	0/2	0/2	0/2
A/duck/Gunma/466/2011 (H7N9)	7.7×10^7	NT	NT	0/2	0/2	0/2
A/duck/Hong Kong/293/1978 (H7N2)	1.4×10^8	NT	NT	0/2	0/2	0/2
A/quail/Aichi/1/2009 (H7N6)	8.2×10^7	NT	NT	0/2	0/2	0/2
A/Netherlands/33/2003 (H7N7)	4.5×10^7	NT	NT	0/2	0/2	0/2

NT indicates "not tested."
[a] Viral RNA copy number was calculated based on the M gene of each virus as described in the text.

tool for the surveillance and early screening of cases infected with the A/H7N9 virus, and specifically for the method to be performed easily in quarantine conditions and clinics should an epidemic of A/H7N9 occur, a new assay was developed to detect the A/H7N9 virus by the RT-LAMP method.

Primer set for the RT-LAMP assay to detect specifically the HA gene of A/H7N9 was designed based on the HA genes of the A/H7N9 viruses deposited in the influenza sequence database of GISAID using Primer Explorer V4 software (Eiken Chemical, Tokyo, Japan) (Table 1). RT-LAMP was carried out using an RNA Amplification Kit (RT-LAMP; Eiken Chemical). The reaction mixture contained 12.5 μL of 2× reaction mix, 1 μL enzyme mix, 4 μL distilled water, 2.5 μL of 10× primer mix (containing 16 μM each of the FIP and BIP primers, 2 μM each of the F3 and B3 primers, and 8 μM each of the LoopF and LoopB primers), and 5 μL template RNA. The mixture was incubated using a Loopamp Realtime Turbidimeter (LA-320C; Eiken Chemical) for 35 min at 62.5 °C and then for 5 min at 80 °C to terminate the reaction.

Seasonal influenza A and B viruses isolated from humans by using Madin–Darby canine kidney (MDCK) cells, namely, A/Uruguay/716/2007 (H3N2), A/Perth/16/2009 (H3N2), A/Narita/1/2009 (H1N1)pdm09, A/California/07/2009 (H1N1)pdm09, B/Florida/4/2006, B/Brisbane/60/2008, and B/Massachusetts/2/2012, were used to test the specificity of the RT-LAMP assay. The H5 subtype avian influenza A viruses A/chicken/Ibaraki/1/2005 (H5N2) and A/whooper swan/Hokkaido/4/2011 (H5N1) and H7 subtype avian influenza A viruses (listed in Table 2) isolated using embryonated eggs were also used. RNA was prepared from 140 μL of the culture medium of MDCK cells or the allantoic fluid of embryonated eggs using a QIAamp® Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The yield of the purified viral RNA was checked by performing a type A real-time RT-PCR (rRT-PCR) assay for all viruses, and RNA copy number was calculated based on the M gene, as described previously (Nakauchi et al., 2011a). To evaluate the specificity of the assay, eight nasal swabs suspended in virus transport medium (two nasal swabs confirmed to be positive for seasonal influenza A (H3N2), two positive for influenza A (H1N1pdm09), two positive for influenza B viruses, and two negative for both influenza viruses by real-time RT-PCR) were also used. RNA was extracted from 50 μL of the nasal swabs suspended in virus transport medium using a MagMAX™ 96 Viral Isolation Kit (Ambion, Austin, TX) with KingFisher Flex (Thermo Fisher Scientific, Waltham, MA) according to the manufacturers' instructions. The RT-LAMP assay did not cross-react with seasonal influenza A (H3N2 and H1N1pdm09) or influenza B viruses isolated from humans, with highly pathogenic avian influenza A (H5N1) viruses, with nasal swabs positive for seasonal influenza A (H3N2 or H1N1pdm09) or influenza B, or with nasal swabs negative for both influenza viruses (data not shown). The RT-LAMP assay also did not cross-react with five out of six H7 subtype avian influenza A viruses belonging to the Eurasian lineage (Table 2) (Kageyama et al., 2013). The assay reacted slightly with A/duck/Fukui/1/2004 (H7N7); however, its sensitivity was approximately two orders of magnitude lower than that for A/Anhui/1/2013 (Table 2). On the basis of phylogenetic analysis (Takayama et al., under preparation), it was shown that the HA gene of A/duck/Fukui/1/2004 was closest to that of A/Anhui/1/2013 within the six Eurasian lineage H7 subtype avian influenza A viruses used in this study. As the RT-LAMP target region of the HA gene for A/Anhui/1/2013 showed the highest identity with that for A/duck/Fukui/1/2004 (five out of eight regions showed 100% similarity between these viruses, Fig. 1 and Table 2), the RT-LAMP assay may react slightly with A/duck/Fukui/1/2004. Given that the RT-LAMP assay showed little or no cross-reactivity with other Eurasian lineage H7 subtype avian influenza A viruses, the assay developed in this study is highly specific for the detection of the H7 gene of the A/H7N9 virus isolated from humans in China and is able to discriminate the A/H7N9 virus from not only seasonal influenza A and B viruses circulating in humans but also highly pathogenic avian influenza A (H5N1) viruses.

To determine the detection limit of the RT-LAMP assay, RT-PCR was used to amplify the full-length HA gene of A/Anhui/1/2013(H7N9), and the resulting PCR product containing

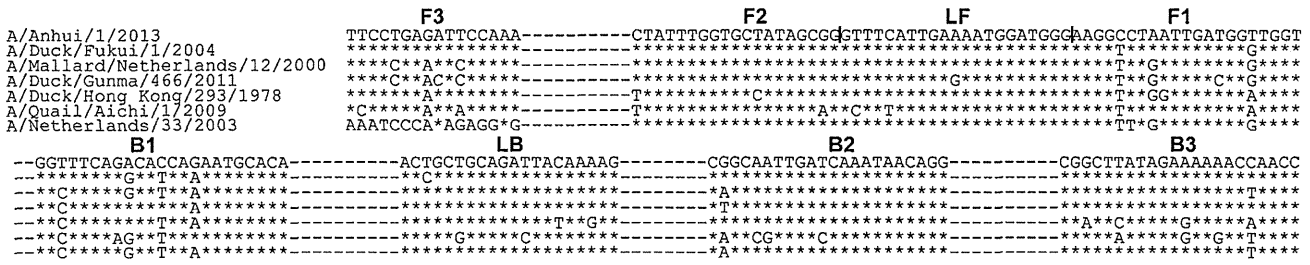

Fig. 1. Nucleotide sequence alignment of the RT-LAMP target region of the HA gene. The F1, F2, F3, LF, B1, B2, B3, and LB regions are indicated above the sequences.

Table 3
Detection limits of the RT-LAMP assay using serial dilutions of *in vitro* transcribed control viral RNA.

Template RNA concentration (copies/reaction)	Number of positive replicates/number of tests for each assay (positive %)
500	6/6 (100)
50	6/6 (100)
5	1/6 (16.7)
0.5	0/6 (0)

the T7 promoter was then transcribed *in vitro*. The detailed procedure is described below. The primer Uni12 (5′-AGCAAAAGCAGG-3′) (Hoffmann et al., 2001) was used for RT with a SuperScript® III Reverse Transcriptase Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The HA gene of A/Anhui/1/2013 was amplified using the following primer pair: H7HA-PC-SP6R (5′-ATTTAGGTGACACTATAGAAAGCAAAAGCAGGGGATA-3′) and H7HA-PC-T7F(5′-TAATACGACTCACTATAGGGAGTAGAAACAAGGGTGT-3′). PCR was performed using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific). The PCR product was gel purified using a QIAquick Gel Extraction Kit (Qiagen) and transcribed using a MegaScript T7 *in vitro* transcription kit (Thermo Fisher Scientific) according to the manufacturers' instructions. After DNase digestion to remove residual RT-PCR products, the transcribed RNA was purified twice using the TRIzol Reagent (Thermo Fisher Scientific) and quantified by spectrophotometric analysis. The detection limit of the RT-LAMP assay was 42.47 copies/reaction, as determined by performing serial dilutions of *in vitro*-transcribed control viral RNA for 6 replicates and then calculating the concentration where there was 95% positivity using the results shown in Table 3. The detection limit of the assay was highly sensitive and almost comparable to the general detection limit of rRT-PCR.

The assay developed in this study was shown to be highly specific and sensitive for detecting the A/H7N9 virus by using isolated viruses and may be applicable to direct RT-LAMP methods (Nakauchi et al., 2011b). Recently, a one-step rRT-PCR assay was developed to detect the A/H7N9 virus (Takayama et al., under preparation). In Japan, manuals prepared by the National Institute of Infectious Diseases for the use of these methods (including the RT-LAMP method) to detect the A/H7N9 virus have been shared with 74 prefectural and municipal public health institutes and 16 quarantine stations, and diagnosis systems have been established to detect cases infected with the A/H7N9 virus in preparation for the influenza season.

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Real-time RT-PCR assays for discriminating influenza B virus Yamagata and Victoria lineages

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ABSTRACT

Since the late 1980s, two genetically and antigenically distinct lineages of influenza B virus, namely, B/Victoria/2/87-like (B/Victoria) and B/Yamagata/16/88-like (B/Yamagata), have co-circulated. In this study, one-step real-time reverse transcription-PCR (rRT-PCR) assays were developed to differentiate B/Victoria and B/Yamagata lineages. The assays were evaluated using *in vitro* transcribed control RNA, isolated viruses, and other respiratory pathogenic viruses, and were shown to have high sensitivity, good linearity ($R^2 = 0.99$), and high specificity. Using the developed rRT-PCR assays, 169 clinical specimens collected between 2010 and 2013 were then tested, resulting in the identification of 20 clinical specimens as positive for influenza B virus. Of these, 14 and 6 samples were identified as positive for the B/Victoria and B/Yamagata lineages, respectively, whereas 149 samples were negative for the influenza B virus. The rRT-PCR assays were also examined using 20 clinical isolates from 20 influenza B virus-positive specimens, revealing that there was no discrepancy between the results from the rRT-PCR assays and the hemagglutination inhibition (HI) test, with the exception that one clinical isolate with different antigenicity could not be discriminated by the HI test. The present results suggest that these highly sensitive and specific assays are useful not only for diagnosing influenza viruses but also for their surveillance.

1. Introduction

Influenza A, B, and C viruses are members of the *Orthomyxoviridae* family of RNA viruses, circulate in humans, and cause respiratory illness every year (Wright et al., 2007). Influenza A viruses comprise a number of subtypes, whereas influenza B and C viruses are not divided into subtypes (Wright et al., 2007). Since the late 1980s, two genetically and antigenically distinct lineages of influenza B virus, namely, B/Victoria/2/87-like (B/Victoria lineage) and B/Yamagata/16/88-like (B/Yamagata lineage), have co-circulated (Kanegae et al., 1990; Rota et al., 1990). In Japan, the majority of circulating influenza B viruses during the 2010/2011 influenza season was of the B/Victoria lineage. During the 2011/2012 and 2012/2013 seasons, both lineages co-circulated; the B/Victoria lineage was dominant in the 2011/2012 season, whereas the B/Yamagata lineage was dominant in the 2012/2013 season (http://www.who.int/influenza/gisrs_laboratory/flunet/en/). The trivalent vaccines currently licensed for use in Japan include only one lineage of the influenza B virus; therefore, it is important to conduct surveillance to know which lineage is predominant at any one time to select a candidate virus for the generation of vaccines.

Conventionally, the two lineages of the influenza B virus are differentiated antigenically by the hemagglutination inhibition (HI) test with immune sera raised against each lineage. The HI test is a conventional method to define the type, subtype, and lineage of clinical isolates; however, it is a time-consuming and complicated process that includes the isolation of viruses from clinical specimens and the preparation of immune sera. Recently, PCR-based assays for the rapid diagnosis of influenza viruses have become the gold standard and many real-time RT-PCR (rRT-PCR) assays have been reported (Wang and Taubenberger, 2010). Compared with the HI test, rRT-PCR assays have the advantage of being able to be performed rapidly, sensitively, and specifically using purified RNA from clinical specimens without requiring a viral isolation step.

In Japan, several rRT-PCR assays for typing influenza A and B viruses and subtyping influenza A viruses were developed using minor groove binder (MGB) probes; the assay scheme was established and shared with 74 prefectural and municipal public health

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Table 1
Primers and probes.

Names	Sequences (5′–3′)	Orientation	Position[a]
Primers and probe for B/NS rRT-PCR			
NIID-Type B TMPrimer-F1	GGAGCAACCAATGCCAC	+	43–59
NIID-Type B TMPrimer-R1	GTKTAGGCGGTCTTGACCAG	–	138–147
NIID-Type B Probe 1	(FAM)ATAAACTTTGAAGCAGGAAT(MGB)	+	61–80
Primers and probe for B/Vic rRT-PCR			
F3vic v2	CCTGTTACATCTGGGTGCTTTCCTATAATG	+	310–339
R3vic v2	GTTGATARCCTGATATGTTCGTATCCTCKG	–	378–407
FAM-Type B HA Victoria	(FAM)TTAGACAGCTGCCTAACC(MGB)	+	356–373
Primers and probes for B/Yam rRT-PCR			
F3yam v2	CCTGTTACATCCGGGTGCTTYCCTATAATG	+	310–339
R3yam v2	GTTGATAACCTKATMTTTTCATATCCTCTG	–	378–407
FAM-Type B HA Yamagata	(FAM)TCAGGCAACTASCCAATC(MGB)	+	356–373
VIC-Type B HA Yamagata[b]	(VIC)TCAGGCAACTASCCAATC(MGB)	+	356–373
Sequencing primers			
BHA1-N	AATATCCACAAAATGAAGGC	+	Noncoding-1–8
BHA1-187F	TGCAAATCTCAAAGGAACAA	+	168–187
BHA1-400R	GTCCTCCTGGTGCCTTTTCT	–	426–445
BHA1-703-722F	CCTCAAAAGTTCACCTCATC	+	673–692
BHA1-802R	GCACCATGTAATCAACAACA	–	783–802
BHA1-C	AGCAATAGCTCCGAAGAAAC	–	1107–1088

[a] The nucleotide positions of the NS and HA genes are based on cRNA sequences obtained from the GISAID database. The isolate ID number for the NS gene of B/Florida/4/2006 is EPI_ISL_22808, and those for the HA genes of B/Brisbane/60/2008 and B/Massachusetts/02/2012 are EPI_ISL_24365 and EPI_ISL_117042, respectively.
[b] The VIC-labeled probe was used for the duplex one-step rRT-PCR assay.

institutes for the diagnosis and surveillance of influenza viruses (Nakauchi et al., 2011b). However, rRT-PCR assay for universally detecting influenza B viruses, whose target region was the non-structural protein (NS) gene (B/NS rRT-PCR; the sequences of the primers and probe are listed in Table 1), was not able to discriminate between the B/Victoria and B/Yamagata lineages.

An rRT-PCR assay for discriminating the B/Victoria and B/Yamagata lineages was first reported by Biere et al. (2010). However, the sequences of the forward primer and the two probes used in the study of Biere et al. had low homology with the recent influenza B viruses circulating in Japan. Another rRT-PCR assay for discriminating these two lineages was reported by Zhang et al. (2012); however, this method did not use MGB probes, resulting in different conditions for this assay compared to the assay used in Japan.

To monitor rapidly and simply the circulating situation of these two lineages of influenza B virus and as a complement to the HI test, rRT-PCR assays for discriminating the B/Victoria and B/Yamagata lineages (B/Vic rRT-PCR and B/Yam rRT-PCR, respectively) were developed by using MGB probes in this study. The highly sensitive and specific rRT-PCR assays for detecting and discriminating these two lineages of influenza B virus will be useful for the diagnosis and surveillance of influenza B viruses.

2. Materials and methods

2.1. Primer and probe design

The nucleotide sequences of the hemagglutinin (HA) gene from influenza B viruses posted over the past 5 years in the Global Initiative on Sharing Avian Influenza Data (GISAID) database were aligned using ClustalW software (Larkin et al., 2007). On the basis of the sequences of the HA gene, 2 probes were designed to discriminate between the B/Victoria and B/Yamagata lineages in which 6 nucleotides (nt) out of the 18 nt probe sequence were different between these lineages. The sequences and positions of the primers and probes are listed in Table 1.

2.2. Viruses

All influenza viruses included in this study were isolated using Madin–Darby Canine Kidney (MDCK) cells. In addition to B/Florida/4/2006 (B/Yamagata), B/Brisbane/60/2008 (B/Victoria), and B/Massachusetts/2/2012 (B/Yamagata), several influenza A viruses were used to validate the specificity of the assays: A/Uruguay/716/2007 (H3N2), A/Perth/16/2009 (H3N2), A/Narita/1/2009 (H1N1)pdm09, and A/California/07/2009 (H1N1)pdm09.

The following the viral respiratory pathogens (the clinical isolates) were used to validate the specificity of the assays: respiratory syncytial viruses A and B; human parainfluenza virus types 1–4; human rhinovirus types A and B; human metapneumovirus types A1 and B2; human coronaviruses OC43, 229E, HKU1, and NL63; human bocavirus; human enterovirus; and human adenoviruses 2 and 4.

2.3. Preparation of RNA transcript controls

To construct an RNA-positive control for the B/NS, B/Vic, and B/Yam rRT-PCR assays, each target gene segment (described in Table 2) was amplified by RT-PCR, and the resulting PCR product, containing the T7 promoter, was then transcribed *in vitro*. The procedure is described in detailed below.

The primer Uni8 (5′-GCAGAAGC-3′) (Zou, 1997) was used for RT using a SuperScript® III Reverse Transcriptase Kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. The NS gene segment of B/Florida/4/2006 was amplified using the following primer pair: TypeB-NS-F(−22–6) (5′-TAGTCACTGGCAAACAGGAAAAATGGCG-3′) and TypeB-NS-R (+1–+29)+T7 (5′-TAATACGACTCACTATAGGGGTAGTAACAAGAGG-ATTTTTATTTTAAAT-3′). The HA gene segments of the B/Brisbane/

Table 2
Detection limits of each assay using serial dilutions of *in vitro* transcribed control viral RNA.

Template RNA concentration (copies/reaction)	Number of positive replicates/number of tests for each assay (positive %)		
	B/NS[a]	B/Vic[b]	B/Yam[c]
10	6/6 (100)	6/6 (100)	6/6 (100)
5	6/6 (100)	6/6 (100)	6/6 (100)
1	0/6 (0)	1/6 (16.7)	3/6 (50)
0.1	0/6 (0)	0/6 (0)	0/6 (0)

The template RNA for each assay was the NS gene segment of [a] B/Florida/4/2006 and the HA gene segments of [b] B/Brisbane/60/2008 and [c] B/Massachusetts/02/2012.

60/2008 (B/Victoria lineage) and B/Massachusetts/2/2012 (B/Yamagata lineage) viruses were amplified using the following primer pairs: FLUB Vic HA-F (5′-ATGAAGGCAATAATTGTACTAC-TCATGGTAGTAACATCC-3′) and FLUB HA-R T7 (5′-TAATACGA-CTCACTATAGGGTTATAGACAGATGGAGCA-3′), and FLUB Yam HA-F (5′-ATGAAGGCAATAATTGTACTACTAATGGTAGTAACATCC-3′) and FLUB HA-R T7, respectively. PCR was performed using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific). The PCR product was gel purified using a QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) and transcribed using the T7 RiboMAX™ Express Large Scale RNA Production System (Promega, Madison, WI) according to the manufacturers' instructions. After TURBO® DNase (Thermo Fisher Scientific) digestion to remove the residues of the RT-PCR products, the transcribed RNA was purified twice using the TRIzol Reagent (Thermo Fisher Scientific), and then the dNTPs and NTPs were removed using MicroSpin G-25 Columns (GE Healthcare, Piscataway, NJ) according to the manufacturer's instructions. The purified transcribed RNA was quantified by spectrophotometric analysis and its integrity was assessed with a 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA). Total RNA was prepared as described in Section 2.6.

2.4. Sequencing of the partial HA gene of a clinical isolate

To confirm the lineage of a clinical isolate, the HA1 region of the HA gene of B/Tokyo/F11-015/2011 was sequenced. To amplify the HA1 region, using extracted RNA, RT and PCR were carried out as described in Section 2.3 with the paired primers BHA1-N and BHA1-C (listed in Table 1) for PCR. The PCR products were purified and then sequenced using 6 primers (listed in Table 1). Total RNA was prepared as described in Section 2.6.

2.5. Clinical specimens

Nasal and/or pharyngeal swabs collected from patients with influenza-like illness and suspended in a UTM 360 C Kit (Copan, Brescia, Italy) were obtained from Showa General Hospital, Japan. These specimens were collected between 2010 and 2013. The study protocol was approved by the Ethics Committee at NIID, and the study was performed in compliance with the Declaration of Helsinki. Informed consent was obtained from all patients.

2.6. RNA preparation

Supernatants from cultured MDCK cells were centrifuged at 10,000 × *g* for 10 min. Viral RNA was prepared from 140 μL of the supernatant using a QIAamp® Viral RNA Kit (QIAGEN) according to the manufacturer's instructions with the slight modification that the viral RNA was eluted in 70 μL AVE buffer (QIAGEN).

Total RNA was prepared from the clinical specimens using a QIAamp® Viral RNA Kit (QIAGEN) (using 140 μL of clinical specimen) or MagMAX™ 96 Viral Isolation Kit (Thermo Fisher Scientific) (using 50 μL of clinical specimen) with KingFisher Flex (Thermo Fisher Scientific) according to the manufacturers' instructions. Total RNA from the 140-μL clinical specimens was eluted with 60 μL AVE buffer (QIAGEN), whereas total RNA from the 50-μL clinical specimens was eluted with 30 μL elution buffer (Thermo Fisher Scientific).

2.7. One-step rRT-PCR assay

The reaction was performed using a QuantiTect® Probe RT-PCR Kit (QIAGEN) according to the manufacturer's instructions. Briefly, the 25 μL assay contained 12.5 μL of 2× QuantiTect Probe PCR Master Mix, 0.25 μL QuantiTect RT Mix, 0.1 μL RNase Inhibitor (Thermo Fisher Scientific), 1.5 μL of 10 μM forward primer, 1.5 μL of 10 μM reverse primer, 0.5 μL of 5 μM probe, 3.65 μL distilled water, and 5 μL RNA template. Cycling was performed as follows: 30 min at 50 °C to activate RT, followed by an initial denaturation step for 15 min at 95 °C and 45 cycles of amplification (denaturation at 95 °C for 15 s and annealing as well as extension at 56 °C for 75 s) using a LightCycler® 480 (Roche Molecular Biochemicals, Basel, Switzerland). Fluorescent signals were collected during the annealing and extension steps, and the amplification data were analyzed using Light Cycler® 480 SW1.5 software according to the manufacturer's instructions. The rRT-PCR assays for typing influenza A and B viruses and subtyping H1pdm and H3 (Nakauchi et al., 2011b) were also performed for the diagnosis of the clinical specimens.

2.8. One-step duplex rRT-PCR assay

The reaction was performed using a QuantiTect® Virus + ROX Vial Kit (QIAGEN) according to the manufacturer's instructions and as described previously (Nakauchi et al., 2011a). For testing the clinical isolates using cultured medium, the 20 μL assay contained 4 μL of 5× QuantiTect Virus NR Master Mix, 0.2 μL QuantiTect Virus RT Mix, 1.2 μL each of two 10 μM forward primers, 1.2 μL each of two 10 μM reverse primers, 0.4 μL each of two 5 μM probes, 7.6 or 8 μL distilled water, and 2 μL culture medium. For testing the clinical specimens using extracted RNA, 5 μL of RNA template were used. Cycling was performed as follows: 20 min at 50 °C to activate RT, followed by an initial denaturation step for 5 min at 95 °C and 45 cycles of amplification (denaturation at 95 °C for 15 s and annealing as well as extension at 56 °C for 45 s) using a LightCycler® 480 (Roche Molecular Biochemicals). Fluorescent signals were collected during the annealing and extension steps, and the amplification and endpoint data were analyzed using Light Cycler® 480 SW1.5 software according to the manufacturer's instructions.

2.9. HI test

Ferret antisera raised against MDCK-grown B/Brisbane/60/2008 (B/Victoria lineage) and B/Massachusetts/2/2012 (B/Yamagata lineage) viruses were treated with RDEII (Denka Seiken Co., Tokyo, Japan) to remove nonspecific inhibitors. A 1:10 dilution of the treated sera was prepared with phosphate-buffered saline. Two-fold serial dilutions of sera were mixed with 4 HA units of antigen virus per well and preincubated for 60 min in 96-well plates at room temperature; 0.5% turkey red blood cells were added, and the plate was incubated for 45 min at room temperature. HI titers of the sera were determined as the highest dilution that did not display hemagglutinating activity (http://apps.who.int/iris/bitstream/10665/44518/1/9789241548090_eng.pdf:1-153).

3. Results

3.1. Development of the rRT-PCR assays

The specificity of the B/NS rRT-PCR assay for detecting both lineages of influenza B virus, and the B/Vic and B/Yam rRT-PCR assays for discriminating the two lineages of influenza B virus were evaluated using B/Victoria, B/Yamagata, A/H3N2, and A/H1N1pdm influenza viruses and other viral respiratory pathogens. The B/NS rRT-PCR assay reacted specifically with both lineages of influenza B virus (data not shown). The B/Vic rRT-PCR assay reacted specifically with B/Victoria, but not with B/Yamagata, whereas the B/Yam rRT-PCR assay reacted specifically with B/Yamagata, but not with B/Victoria (data not shown). All three assays showed no cross-reactivity against influenza A viruses or other viral respiratory pathogens (data not shown).

The detection limit of each assay was determined by performing serial dilutions of *in vitro* transcribed control viral RNA for 6

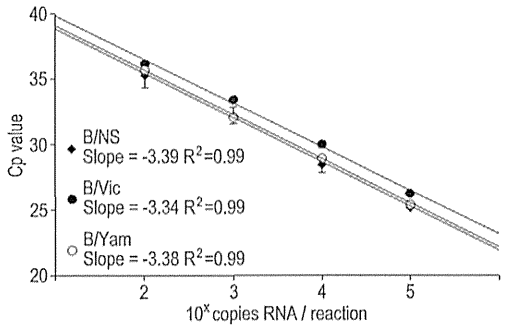

Fig. 1. Standard curves of each assay. Ten-fold serial dilutions of synthesized RNA were used for each rRT-PCR assay performed with six replicates. The standard curves were generated using the average Cp values obtained from the assay performed with six replicates. The correlation coefficients and slope of the standard curves are represented in the graph. The standard curves were made based on the B/NS rRT-PCR assay performed using a synthesized NS gene segment of B/Florida/4/2006 (diamonds) and the B/Vic and B/Yam rRT-PCR assays performed using a synthesized HA gene segment of B/Brisbane/60/2008 (filled circles) and B/Massachusetts/02/2012 (open circles), respectively.

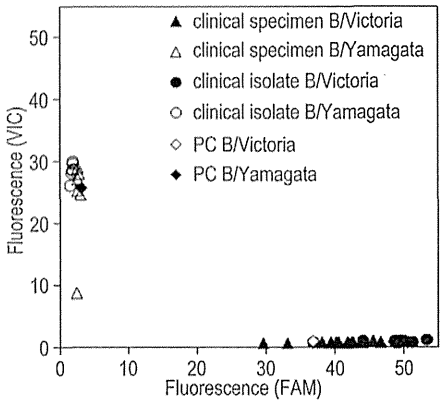

Fig. 2. Endpoint fluorescence plot of the duplex rRT-PCR assay using clinical specimens and isolates. The relative fluorescence of B/Victoria (FAM) and B/Yamagata (VIC) is plotted on the *x*-axis and *y*-axis, respectively. The clinical specimens discriminated as B/Victoria or B/Yamagata are represented as filled or open triangles, and the clinical isolates discriminated as B/Victoria or B/Yamagata are represented as filled or open circles, respectively. One hundred copies each of the synthesized HA gene segment of B/Brisbane/60/2008 (PC B/Victoria, open diamond) and B/Massachusetts/2/2012 (PC B/Yamagata, filled diamond) were used as positive controls for the assay.

replicates in each assay. The detection limits of the B/NS, B/Vic, and B/Yam rRT-PCR assays were determined to be 8.27, 4.31, and 3.85 copies/reaction, respectively, calculated by a Probit analysis using the results shown in Table 2. A standard curve of each assay was also generated (Fig. 1). The standard curve showed a linear relationship between the log of the viral titer and the crossing point (Cp) value for all assays (Fig. 1). The correlation coefficient of the standard curve was 0.99 for all assays, indicating a precise log–linear relationship between the viral titer and Cp value (Fig. 1).

3.2. Validation of the rRT-PCR assays using clinical specimens

From 2010 to 2013, clinical specimens (nasal and/or pharyngeal swabs) from patients with an influenza-like illness ($n = 169$) were obtained, and the B/NS rRT-PCR assay was performed on these samples. As a result, 20 out of 149 samples were identified as positive for influenza B virus. Twenty B/NS-positive samples were then tested using the B/Vic and B/Yam rRT-PCR assays, revealing that 14 and 6 samples were identified as positive for the B/Victoria and B/Yamagata lineages, respectively.

3.3. Validation of the rRT-PCR assays using clinical isolates

Clinical isolates from 20 influenza B virus-positive clinical specimens (listed in Table 3) were tested using the B/NS, B/Vic, and B/Yam rRT-PCR assays and also by the conventionally performed HI test. As shown in Table 3, 14 and 6 clinical isolates were identified as positive for the B/Victoria and B/Yamagata lineages, respectively, consistent with the results of the three rRT-PCR assays on the original clinical specimens. Antiserum against B/Massachusetts/2/2012 (B/Yamagata) reacted with all 6 B/Yam-positive clinical isolates. Antiserum against B/Brisbane/60/2008 (B/Victoria) reacted with 13 B/Vic-positive clinical isolates; however, it reacted poorly with one B/Vic-positive clinical isolate, B/Tokyo/F11-015/2011, with 8-fold-reduced HI titers from the homologous titer. To identify the cause of the low reaction of the antiserum against B/Brisbane/60/2008 with B/Tokyo/F11-015/2011, the HA1 region (1–341 amino acids) of the HA gene was sequenced and compared with the HA1 region of B/Brisbane/60/2008. This revealed that B/Tokyo/F11-015/2011 has 8 amino acid mutations (N26D, T52I, K90N, I161V, K180N, K182R, P187S, and A217T).

3.4. Application of the B/VIC and B/Yam RT-PCR assays to the duplex rRT-PCR assay

To facilitate an assay to discriminate between the two lineages, the primers and probes for the B/VIC and B/Yam rRT-PCR assays were mixed and the duplex rRT-PCR assay was constructed. For the duplex rRT-PCR assay, the probe for B/Victoria was labeled with FAM and the probe for B/Yamagata was labeled with VIC (as listed in Table 1). Using 20 clinical specimens and 20 clinical isolates that were identified as positive for influenza B virus, the duplex rRT-PCR assay was evaluated. For the clinical isolates, the assay was performed by using the cultured supernatants from infected MDCK cells directly, without RNA purification, as the template of the assay for screening a large number of clinical isolates. For the clinical specimens, purified RNA was used for the assay. As shown in Fig. 2, 14 and 6 clinical specimens were determined as B/Victoria and B/Yamagata, respectively. Similarly, 14 and 6 clinical isolates were determined as B/Victoria and B/Yamagata, respectively (Fig. 2).

4. Discussion

The B/NS rRT-PCR assay used for detecting influenza B viruses and the B/Vic and B/Yam rRT-PCR assays used for discriminating the two lineages of the influenza B virus, were shown to have good linearity ($R^2 = 0.99$) and high sensitivity (Table 2 and Fig. 1). No cross-reactivity or nonspecific reactions were observed in any of the assays performed using isolated viruses and clinical specimens (data not shown). In the assay performed using clinical specimens, 20 samples were identified as positive for influenza B virus and 149 samples were negative in the B/NS rRT-PCR assay. Of the 149 B/NS-negative samples, 81 and 3 samples were identified as positive for A/H3 and A/H1pdm by performing the previously developed Type A, H1pdm, and H3 rRT-PCR assays (Nakauchi et al., 2011b), and no influenza A or B viruses were detected in the remaining 65 samples. All 20 B/NS-positive clinical samples were discriminated either as B/Victoria or B/Yamagata by the B/Vic and B/Yam rRT-PCR assays, respectively. These results suggest that these three rRT-PCR assays are highly sensitive and specific for each target gene and should thus be of great use for detecting and discriminating the two lineages of the influenza B virus.

Table 3
Comparing the HI test and rRT-PCR assays using clinical isolates.

Influenza B virus	HI titer[a] of postinfection ferret antisera		rRT-PCR[b]		
	B/Brisbane/60/2008	B/Massachusetts/2/2012	B/NS	B/Vic	B/Yam
Reference virus					
B/Brisbane/60/2008	**80**	<10	+	+	–
B/Massachusetts/2/2012	<10	**160**	+	–	+
Clinical isolate (test virus)					
B/Tokyo/SH-F11-015/2011	10	<10	+	+	–
B/Tokyo/SH-F11-026/2011	40	<10	+	+	–
B/Tokyo/SH-F11-030/2011	80	<10	+	+	–
B/Tokyo/SH-F11-031/2011	80	<10	+	+	–
B/Tokyo/SH-F11-032/2011	160	<10	+	+	–
B/Tokyo/SH-F12-068/2012	160	10	+	+	–
B/Tokyo/SH-F12-082/2012	10	160	+	–	+
B/Tokyo/SH-F12-099/2012	160	<10	+	+	–
B/Tokyo/SH-F12-103/2012	10	160	+	–	+
B/Tokyo/SH-F12-138/2012	160	<10	+	+	–
B/Tokyo/SH-F12-143/2012	10	160	+	–	+
B/Tokyo/SH-F12-155/2012	160	<10	+	+	–
B/Tokyo/SH-F12-157/2012	160	<10	+	+	–
B/Tokyo/SH-F12-159/2012	160	<10	+	+	–
B/Tokyo/SH-F12-161/2012	<10	160	+	–	+
B/Tokyo/SH-F12-165/2012	160	<10	+	+	–
B/Tokyo/SH-F12-170/2012	160	<10	+	+	–
B/Tokyo/SH-F12-173/2012	160	<10	+	+	–
B/Tokyo/SH-F13-066/2013	10	160	+	–	+
B/Tokyo/SH-F13-068/2013	10	160	+	–	+

[a] The underlined text indicates the homologous titers of the antisera.
[b] "+" and "–" indicate "positive" and "negative" for each rRT-PCR assay.

The results of the HI test and the B/Vic and B/Yam rRT-PCR assays were compared using 20 clinical isolates (Table 3) from 20 influenza B virus-positive clinical samples. The results of rRT-PCR assays corresponded to those of the HI test with one exception. B/Tokyo/F11-015/2011 and its original specimen were identified as positive for the B/Victoria lineage by the B/Vic and B/Yam rRT-PCR assays; however, antiserum against B/Brisbane/60/2008 reacted poorly with B/Tokyo/F11-015/2011. It was found that B/Tokyo/F11-015/2011 has eight different amino acids, namely, N26D, T52I, K90N, I161V, K180N, K182R, P187S, and A217T, in the HA1 region when compared with B/Brisbane/60/2008. The amino acid positions at 182 and 217, where B/Tokyo/F11-015/2011 has mutations, were reported as antigenic sites by analyzing escape mutants from monoclonal antibodies (Tung et al., 2004), and the Thr to Ile mutation at position 52 is also known to affect antigenicity (unpublished data from the Influenza Surveillance Group of Japan). Considering these data, B/Tokyo/F11-015/2011 is antigenically different from B/Brisbane/60/2008; thus, the HI test could not identify B/Tokyo/F11-015/2011 as B/Victoria.

The HI test has been performed conventionally for determining type, subtype, and lineage of influenza viruses and for detecting antigenic differences between reference and test viruses. The HI test is a very useful method for monitoring the antigenicity of circulating influenza viruses in humans to select a virus for vaccine production; however, the HI test is not able to detect influenza viruses when the test virus is antigenically different from the reference virus. The combination of the B/NS, B/Vic, and B/Yam rRT-PCR assays developed in this study was able to detect and discriminate both lineages of the influenza B virus, including a virus that was undetected by the HI test, with high sensitivity and specificity (Table 3); however, the rRT-PCR assays cannot be used to compare antigenic differences. Therefore, it is important to use the rRT-PCR assays in cooperation with the HI test for conducting surveillance of influenza B viruses.

To facilitate the assay scheme of the B/Vic and B/Yam rRT-PCR assays, a duplex rRT-PCR assay for discriminating the two lineages of the influenza B virus was also constructed. No discrepancy was found between the results from the duplex rRT-PCR assay and the B/Vic and B/Yam rRT-PCR assays for 20 clinical specimens and 20 clinical isolates (Table 3 and Fig. 2). It is noteworthy that the duplex assay could be performed using clinical isolates without an RNA purification step, since a sufficient amount of viral RNA may be released from the virion under the RT conditions (50 °C, 20 min). When considering the handling of a large number of clinical isolates, this assay can be performed rapidly and simply with a lower risk of cross-contamination because an RNA purification step is unnecessary.

In conclusion, the highly sensitive and specific B/NS, B/Vic, and B/Yam rRT-PCR assays developed in this study will be very useful not only for laboratory diagnostic tests but also for speeding up surveillance by discriminating the lineages of influenza B viruses in clinical specimens. Considering that the trivalent vaccines currently licensed for use in Japan include only one lineage of the influenza B virus, the assays are also powerful tools for the rapid recognition of the predominant lineage of influenza B virus at any one time and helpful to decide which candidate virus should be included in vaccines.

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Short communication

Development of an improved RT-LAMP assay for detection of currently circulating rubella viruses

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A B S T R A C T

Rubella virus is the causative agent of rubella. The symptoms are usually mild, and characterized by a maculopapular rash and fever. However, rubella infection in pregnant women sometimes can result in the birth of infants with congenital rubella syndrome (CRS). Global efforts have been made to reduce and eliminate CRS. Although a reverse transcription-loop-mediated isothermal amplification (RT-LAMP) assay for detection of rubella virus has been reported, the primers contained several mismatched nucleotides with the genomes of currently circulating rubella virus strains. In the present study, a new RT-LAMP assay was established. The detection limit of this assay was 100–1000 PFU/reaction of viruses for all rubella genotypes, except for genotype 2C, which is not commonly found in the current era. Therefore, the new RT-LAMP assay can successfully detect all current rubella virus genotypes, and does not require sophisticated devices like TaqMan real-time PCR systems. This assay should be a useful assay for laboratory diagnosis of rubella and CRS.

Rubella virus (RV) is the causative agent of rubella, also known as German measles. RV is the sole member of the genus *Rubivirus* in the family Togaviridae and its genome consists of a single positive-strand RNA of approximately 9.8 kb in length. The viral genome contains two open reading frames (ORFs). The 5′ ORF encodes two non-structural proteins (p150 and p90) (Pugachev et al., 1997; Liang and Gillam, 2000) and the 3′ ORF encodes three structural proteins (E1, E2 and C) (Oker-Blom et al., 1984; Frey, 1994; Yao et al., 1998). Although RV has only one serotype, 13 genotypes have been reported to date, based on analyses of the structural protein gene nucleotide sequences (Abernathy et al., 2011; WHO, 2013). The 13 genotypes are classified into two clades, clade 1 and clade 2. Clade 1 consists of genotypes 1a, 1B, 1C, 1D, 1E, 1F, 1G, 1H, 1I, and 1J, while clade 2 consists of genotypes 2A, 2B, and 2C (WHO, 2013). Genotypes 1E, 1G, 1H, 1J, and 2B were the major RV genotypes circulating worldwide between 2005 and 2010 (Abernathy et al., 2011). Among these, genotypes 1E, 1J, and 2B constitute the majority of RV isolates in East and South-East Asia.

The typical symptoms of rubella are relatively mild, and characterized by a maculopapular rash, short-duration fever, and lymphadenopathy. RV infection of women within the first trimester of pregnancy can cause miscarriage, fetal death, or birth of infants with congenital rubella syndrome (CRS), which includes various birth defects such as sensorineural deafness, heart defects, and cataracts. Rubella is effectively prevented by vaccination with rubella-containing vaccines. In the last two decades, the number of member states of the World Health Organization (WHO) that have introduced rubella-containing vaccines into their national immunization schedules has increased significantly (from 83 in 1996 to 130 in 2009) (WHO, 2011). The WHO Region of the Americas (AMR or PAHO) and European Region (EUR) have established goals to eliminate rubella by 2010 and 2015, respectively. PAHO is verifying rubella and CRS elimination at present. However, in many countries, especially developing countries, RCVs have not yet been introduced and rubella and CRS surveillance has been weak. Thus, a significant number of CRS cases are likely overlooked in these countries (WHO, 2011).

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Table 1
Primers used for the RT-LAMP assay.

	Primers	Genomic positions	Sequence (5′-3′)
NSP	FIP_NSP5	(251–230)+(171–187)	cgtggagtgctgggtgatcactcccaRaagcgggccatc
	BIP_NSP5	(257–274)+(324–306)	tcgcggtatacccgccgcgggtcgatSaggacgtgta
	F3_NSP5	97–115	cggcagttgggtaagagac
	B3_NSP5	395–380	ccgtcctgtggaggca
	Loop F_NSP5	218–196	gcgtgaaHacaggtctgggtatc
	Loop B_NSP5	286–304	gtggggccctaaagaagcc
E1	FIP_E1	(8557–8539)+(8495–8510)	agaggccagctgcgcgtaccgcgcgtgcaccttct
	BIP_E1	(8600–8617)+(8666–8651)	accgcgtgcgaggttgaatgtcggtggggaagcc
	F3_E1	8475–8494	gcatctggaatggcacacag
	B3_E1	8686–8669	ccgcttgtgcgagtagtg
	Loop F_E1	8535–8521	ccagaggagtaggcg
	Loop B_E1	8618–8632	cctgccttcggacac

Since clinical diagnosis of rubella and CRS is often unreliable, laboratory confirmation is critical. The WHO recommends testing for anti-rubella IgM in serum as the standard method for laboratory diagnosis of rubella. However, anti-rubella IgM may be undetectable until a few days after rash and fever appearance (CDC, 2008; WHO, 2008). On the other hand, viral genome detection can be used to confirm rubella in the first few days after rash appearance, since virus is excreted about 1 week before the rash appearance and the peak of its excretion in the oral fluid coincides with rash appearance. Viral genome detection can also be used to confirm CRS (Bellini and Icenogle, 2011). Several methods, including RT-PCR (or RT-nested PCR) (Zhu et al., 2007), real-time RT-PCR (Okamoto et al., 2010), and RT-loop-mediated isothermal amplification (RT-LAMP) (Mori et al., 2006), have been established for detection of the RV genome.

LAMP was developed for amplification of target DNA with a specific primer set and a DNA polymerase with strand displacement activity (Notomi et al., 2000; Nagamine et al., 2002). LAMP has high specificity because at least four specific primers corresponding to six regions are required for the amplification (Notomi et al., 2000; Nagamine et al., 2002). The LAMP reaction is usually carried out under isothermal conditions at about 65 °C for about 1 h. Therefore, the laboratory diagnosis is easily conducted using simple devices. Importantly, the results can be assessed visually without opening of the sample tubes, which decreases the risk of cross-contamination. There are several methods for detection of LAMP amplification products. Turbidity derived from precipitated magnesium pyrophosphate as a by-product of the amplification is generally employed for LAMP detection (Mori et al., 2001). Turbidity can be monitored in real-time using a turbidimeter or detected visually. Detection by color change after addition of hydroxyl naphthol blue (HNB) to the reaction mixture has also been reported (Goto et al., 2009). HNB is a colorimetric reagent for alkaline earth metal ions. The concentration of magnesium ions in the LAMP reaction mixture decreases during amplification. If amplification occurs, the solution including HNB changes from violet to sky-blue. Users can select a detection method according to their situation. An RNA template can also be amplified by LAMP, by combination with a reverse transcription reaction (RT-LAMP) (Notomi et al., 2000). RT-LAMP has been adapted to detect RNA virus infections (Ushio et al., 2005; Ito et al., 2006). Mori et al. (2006) reported previously an RT-LAMP assay for the RV genome using primers targeting the nucleotide sequence of the E1 region, which were designed based on the KRT vaccine strain (genotype 1a) genome sequence. However, the E1 region is variable in its nucleotide sequences, and the original primers display some mismatches with several RV genotypes, especially 1J, 1E, and 2B, the major genotypes recently circulating in Japan (data not shown). This suggests that the RT-LAMP assay using these mismatched primers may show low sensitivity for detection of these RV genotypes. In the present study, to refine the RT-LAMP assay for detection of currently circulating RV genotypes, we designed new primers targeting the 5′-terminal region of the non-structural protein gene encoding p150. The targeted region is adjacent to the primer-targeted region in a TaqMan real-time PCR method reported previously (Okamoto et al., 2010). The performance of the new RT-LAMP assay developed in this study was examined using viral RNAs of all genotypes.

The new RT-LAMP primer set, named the NSP primer set, was designed using an online software program for LAMP primer design (PrimerExplorer; http://primerexplorer.jp/index.html) to target the region of nucleotides 97–395, a highly conserved region among various genotypes. The primer sequences and positions are shown in Table 1. Previously described primers are also shown in Table 1. The RT-LAMP assay was performed using a Loopamp RNA amplification kit (RT-LAMP; EIKEN Chemical, Tokyo, Japan). An aliquot (5 μl) of each RNA was mixed with 40 pmol of FIP and BIP primers, 20 pmol of Loop-F and Loop-B primers, 5 pmol of F3 and B3 primers, 1 μl of enzyme mix, and 12.5 μl of reaction mix in a total volume of 25 μl. The reaction mixture was incubated at 63 °C for 60 min followed by 85 °C for 5 min in a Loopamp real-time turbidimeter (RT-160C; EIKEN Chemical). The assay was performed in triplicate for each sample, and a no-template control, in which the RNA template was omitted, was included in each assay. Test samples were considered positive if the turbidity signal was greater than 0.1 after 60 min. Controls without template gave no turbidity signals typically, by both a measuring device and visually. A 10-fold dilution series of *in vitro* synthesized RNA (nucleotides 1–782 of the TO-336 strain) was prepared as the standard RNAs (from 2.0×10^5 to 2.0×10^{-1} copies/μl) as described previously (Okamoto et al., 2010). Three distinct sets of standard RNA samples (1.0×10^6 to 1 copies/reaction) were prepared totally independently (starting with *in vitro* transcriptions from plasmids) examined to estimate the detection limit of the assay. Although $>10^4$ copies/reaction were detected in all samples, 10^3 copies/reaction were detected in 78% of samples (Table 2). The

Table 2
Detection limit of the RT-LAMP assay using a series of synthesized standard RNA solutions.

Standard RNA (copies/reaction)	Number of positive samples/number of tested samples			Positive (%)
	1	2	3	
10^6	3/3	3/3	3/3	100
10^5	3/3	3/3	3/3	100
10^4	3/3	3/3	3/3	100
10^3	2/3	2/3	3/3	78
10^2	0/3	0/3	1/3	11
10	0/3	0/3	0/3	0
50% detection dose*	2.75	2.75	2.25	2.58**

* Copies of standard RNA for which 50% of reactions were positive ($\log_{10}$ copies/ml).
** Mean value of three trials.

Table 3
Sensitivity of the RT-PCR assay for different genotypes: 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/BEL/63[1a]VAC	1a	P (25.6 ± 5.21)***	P (30.5 ± 3.80)	N (>60.0)	N (>60.0)
RVi/Jerusalem.ISR/75[1B]	1B	P (20.7 ± 3.56)	N (>60.0)	N (>60.0)	N (>60.0)
RVi/Los Angels.USA/91[1 C]	1C	P (22.6 ± 2.52)	P (32.0 ± 2.08)	N (>60.0)	N (>60.0)
Osaka '94	1D	P (24.1 ± 5.21)	N (>60.0)	N (>60.0)	N (>60.0)
Rvi/Dezhou.CHN/02[1E]	1E	P (20.4 ± 2.70)	P (27.4 ± 3.66)	N (>60.0)	N (>60.0)
Rvi/Linqing.CHN/00[1F]	1F	P (23.0 ± 3.07)	N (>60.0)	N (>60.0)	N (>60.0)
Rvi/UGA/20.01[1G]	1G	P (18.4 ± 0.97)	P (25.4 ± 2.87)	N (>60.0)	N (>60.0)
Rvi/Minsk.BLR/28.05[1H]	1H	P (19.7 ± 0.62)	P (26.1 ± 1.03)	N (>60.0)	N (>60.0)
Rvi/Milan.ITA/46.92[1I]	1I	P (22.9 ± 2.81)	P (31.2 ± 6.42)	N (>60.0)	N (>60.0)
Rvi/Miyazaki.JPN/10.01 CRS[1 J]	1J	P (21.7 ± 1.87)	N (>60.0)	N (>60.0)	N (>60.0)
Rvi/Beijing.CHN/80[2A]VAC	2A	P (22.5 ± 2.39)	P (31.1 ± 2.34)	N (>60.0)	N (>60.0)
Rvi/Telaviv.ISR/68[2B]	2B	P (24.8 ± 4.41)	N(>60.0)	N (>60.0)	N (>60.0)
Rvi/Moscow.RUS/67[2 C]	2C	N (>60.0)	N (>60.0)	N (>60.0)	N (>60.0)

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

end-point, at which 50% of the reactions were positive, was calculated by the Reed-Müench method (Reed and Muench, 1938), and was about 380 copies/reaction. This detection limit was higher than that of the TaqMan real-time PCR assay, which could detect all samples at 10 copies/reaction (Okamoto et al., 2010). The sensitivities of the TaqMan and RT-LAMP assays were reported to be similar for detection of Chikungunya virus and Rift Valley virus (Le Roux et al., 2009; Reddy et al., 2012). The sensitivity of the RT-LAMP assay may be lower than that of the TaqMan real-time PCR assay Because of a complicated secondary structure. The RV genome may form a complicated secondary structure owing to its high (~70%) GC contents (Frey, 1994). Next, we examined whether the new RT-LAMP assay could detect the genomes of all 13 RV genotypes. The 13 RVs were propagated with Vero cells or RK-13 cells. The viral RNA was extracted from 140 μl aliquots of serial dilutions of these virus samples using a QIAamp Viral RNA Mini Kit (QIAGEN, Tokyo, Japan) according to the manufacturer's protocol. To assess the sensitivity of the RT-LAMP (NSP primer set) assay for different genotypes, 5 μl of each RNA extract of the 13 RVs was amplified with the assay (Table 3). Some examples of the time course of turbidity measured by a Loopamp real-time turbidimeter were cited in Fig. 1. Test samples were considered positive if amplification was seen in all triplicate reactions, and three independent experiments were repeated. Table 3

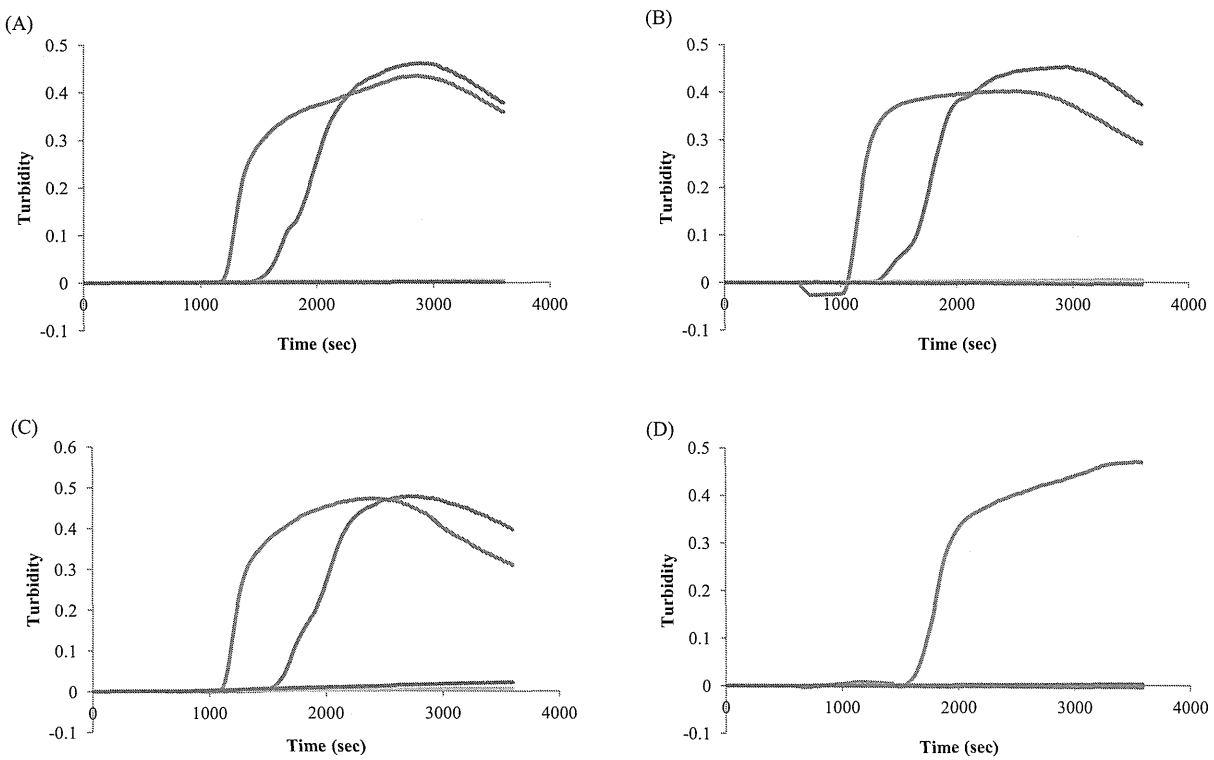

Fig. 1. The time course of turbidity by RT-LAMP assay. The graphs showed the turbidity measured by a Loopamp real-time turbidimeter. Blue, red, green, and violet lines represent 10^3, 10^2, 10, and 1 PFU/reaction of virus strains as follows, respectively. (A) Rvi/Dezhou.CHN/02[1E]; (B) Rvi/Minsk.BLR/28.05[1H]; (C) Rvi/Miyazaki.JPN/10.01 CRS[1J]; (D) Rvi/Telaviv.ISR/68[2B].