Table 2. Antimicrobial resistance of *Salmonella* isolates by food, domestic animals, and humans in Vietnam

Source	No. of examined	No. of resistant isolates[a] (%)										No. of Resistance[b] (%)
		ABPC	SM	KM	GM	OTC	CP	CEZ	CTRX	NA	CPFX	
Food												
Pork	48	3 (6.3)	7(14.6)			10 (20.8)	6 (12.5)					18 (37.5)[c]
Beef	35		2 (5.7)			1 (2.9)	1 (2.9)					2 (5.7)[c,d]
Chicken meat	20	1 (5.0)	4(20.0)	4(20.0)		9 (45.0)	5 (25.0)			7 (35.0)		9 (45.0)[d]
Duck meat	20		3(15.0)	1 (5.0)		3 (15.0)	1 (5.0)			1 (5.0)		4 (20.0)
Shrimp	29					2 (6.9)	1 (3.4)					2 (6.9)
Animal												
Chicken	19					8 (42.1)	8 (42.1)			9 (47.4)		9 (47.4)[e,f]
Duck	25					1 (4.0)	1 (4.0)					1 (4.0)[e]
Pig	26					3 (11.5)	3 (11.5)					3 (11.5)[f]
Human	8					1 (12.5)						1 (12.5)
Total	230	4 (1.7)	16 (7.0)	5 (2.2)	0	38 (16.5)	26 (11.3)	0	0	17 (7.4)	0	49 (21.3)
MIC_{50} (μg/ml)		1	8	2	0.5	2	8	1	<0.125	4	<0.125	
MIC_{90} (μg/ml)		2	8	2	0.5	128	32	1	<0.125	32	<0.125	

a) ABPC: Ampicillin, SM: Streptomycin, KM: Kanamycin, GM: Gentamicin, OTC: Oxytetracycline, CP: Chloramphenicol, CEZ: Cefazolin, CTRX: Ceftriaxone, NA: Nalidixic acid, CPFX: Ciprofloxacin.
b) Isolates resistant to at least one antimicrobial agent.
c, d, e, f) Difference is statistically significant, $P<0.01$.

Table 3. Antimicrobial resistance patterns of *Salmonella* isolates from Vietnam

No. of antimicrobial agents	Resistance pattern[a]	No. of resistant isolates	Serotypes	Source
1	SM	9	*S.* Derby	Pork (7), Beef (1), Duck meat (1)
	OTC	8	*S.* Derby	Shrimp (1)
			S. Anatum	Pork (2), Duck meat (1)
			S. London	Pork (1), Chicken meat (1), Human (1)
			S. Norwich	Pork (1)
	CP	1	*S.* Derby	Pork (1)
	NA	1	*S.* Enteritidis	Chicken (1)
2	ABPC+OTC	1	*S.* Anatum	Pork (1)
	SM+OTC	1	*S.* Hadar	Duck meat (1)
	OTC+CP	8	*S.* Derby	Pork (3), Shrimp (1), Pig (3), Duck (1)
3	ABPC+OTC+CP	3	*S.* Anatum	Pork (2)
			S. Javiana	Chicken meat (1)
	SM+OTC+CP	1	*S.* Weltevreden	Beef (1)
	OTC+CP+NA	11	*S.* Hadar	Chicken meat (1)
			S. Emek	Chicken meat (2), Chicken (8)
4	SM+KM+OTC+NA	3	*S.* Blockley	Chicken meat (1)
			UT	Chicken meat (2)
5	SM+KM+OTC+CP+NA	2	*S.* Blockley	Duck meat (1)
			S. Hadar	Chicken meat (1)
Total		49		

a) ABPC: Ampicillin, SM: Streptomycin, KM: Kanamycin, OTC: Oxytetracycline, CP: Chloramphenicol, NA: Nalidixic acid.

(20.0%) from duck meat, of the 70 isolates from domestic animals, a total of 13 (18.6%) isolates composed by 9 (47.4%) from chicken, 1 (4.0%) from duck, and 3 (11.5%) from pig, and of the 29 isolates from retail shrimp, two (6.9%) isolates, and of the 8 isolates from human, 1 (12.5%) isolate showed resistance to some antimicrobial agents. The resistance rates of *Salmonella* isolates from pork and chicken meat were significantly higher than that from beef ($P<0.01$), and among the isolates from domestic animals, resistant rates from chicken were significantly higher than those from ducks and pigs ($P<0.01$) (Table 2).

Among the resistant isolates, nineteen isolates showed resistance to one antimicrobial agent, 10 to two, 15 to three, 3 to four, and 2 to five antimicrobial agents (Table 3).

All of 17 NA resistant isolates were less susceptible to CPFX (MIC=0.25–2 μg/ml) when compared to the other

isolates.

DISCUSSION

Of the 10 antimicrobial agents analyzed in this study, *Salmonella* isolated in the Mekong Delta, Vietnam showed resistance to 6 agents. In developed countries, high resistance rates have generally been observed against those antimicrobial agents used since early times. In Japan, Miwa *et al.* [20] reported that 182 (63.4%), 184 (64.8%), and 20 (7.0%) among 287 *Salmonella* isolates, originated from chicken carcasses from 1996 to 1999, were resistant to OTC, SM, and ABPC, respectively, and Takahashi *et al.* [25] reported that 100 (80.6%), 95 (76.6%), and 11 (8.9%) among 124 isolates from feces of healthy domestic animals, mainly broiler chicken, in 1999 were resistant to OTC, dehydrostreptomycin (DSM), and ABPC, respectively. White *et al.* [36] reported that in the United States, 36 (80%), 33 (73%), and 12 (27%) among 45 of *Salmonella* isolates from retail ground meat in 1998 were resistant to tetracycline(TC), SM, and ABPC, respectively; and Poppe *et al.* [22] reported that in Canada, 341 (25.5%), 354 (26.5%), and 212 (15.9%) of 1336 isolates from animals, animal food products, and the environment of animal production were resistant to the same respective antimicrobial agents as in the United States. Similarly, resistance to those antimicrobial agents has been reported in developing countries in Southeast Asia. Rasrinaul *et al.* [23] reported that in Thailand, 40 (46%), 12 (14%), and 5 (6%) of 87 *Salmonella* isolates from food samples in 1986 were resistant to TC, SM, and ABPC, respectively. Van *et al.* [32] reported that 91 *Salmonella* isolates from retail raw food samples obtained in Ho Chi Mihn city, Vietnam were resistant to ABPC (22%), amoxicillin (22%), TC (40.7%), KM (2.2%), GM (2.2%), SM (14.3%), sulfafurazole (16.5%), enrofloxacin (8.8%), CP (2.2%), trimethoprim (3.3%) and NA (18.4%). The *Salmonella* isolates from the Mekong Delta, Vietnam also showed resistance to OTC, CP, NA, SM, KM, and ABPC. However, when compared with the results of developed countries and even with the results of neighboring countries, it seems that the resistance rate of *Salmonella* isolates from the Mekong Delta to these antimicrobial agents was relatively low. Furthermore, in the United States, besides resistance to those antimicrobial agents used since early times, resistance to CTRX, a third generation cephalosporin used to treat children with *Salmonella* infection, was detected in 7 (16%) of 45 *Salmonella* isolates in retail ground meat in 1998 [36]. Likewise, Boonmer *et al.* [2] reported that 28 (14.1%) of 199 *Salmonella* isolates from frozen chicken meat in Thailand were also resistant to CTRX. However, CTRX resistant *Salmonella* isolates as well as isolates resistant to relatively new antimicrobial agents were not detected in the Mekong Delta in Vietnam. Van *et al.* [32] also reported that no *Salmonella* isolates originated from retail raw food samples obtained in Ho Chi Mihn city, Vietnam showed resistance to cephalothin, a third generation cephalosporin. This would be partly because intensive livestock production, where antimicrobial agents are used as growth promoter or feed additives, is rare in Vietnam, and most of domestic animals are raised in small farm or farmer yard and fed agricultural by-products.

Moreover, in developed countries, the spread of particular serotypes and phage types that acquired multi-drug resistance has become an increasing public health problem. In the United States, the three most common *Salmonella* serotypes (Typhimurium, Enteritidis, and Newport) accounted for 50% of clinical isolates from human and 44% of clinical animals in 2001, and among them, *S.* Typhimurium and S. Newport have emerged as major multi-drug resistant pathogens [4]. An increase in the incidence of food-borne infections caused by *S.* Enteritidis, and human and animal infections by multi-drug resistant strains of *S.* Typhimurium has been also observed in European countries [3, 6, 31]. Among the multi-drug resistant *Salmonella*, *S.* Typhimurium definitive type DT104 has emerged as a global health problem in human and animal medicine during the last decade because of its resistance to up to nine antimicrobial agents commonly used [8, 13, 26, 27]. Van *et al.* [32] reported that 20.9% of 91 *Salmonella* isolates from retail raw food samples showed multi-drug resistance. In the Mekong Delta, many of the above serotypes have also been isolated, including *S.* Typhimurium, but no resistant strain of this serotype was detected in this study. On the other hand, *S.* Blockley, *S.* Hadar, and *S.* Emek showed a higher rate of multi-drug resistance. Those multi-drug resistant isolates were mainly originated from chicken, chicken meat and duck meat. It is not clear why mainly chicken related isolates showed a tendency to multi-drug resistance, but it might be associated with recent introduction of some commercial chicken farms using feed sold by the United States or European feed companies, in which antimicrobial agents are including in the feed.

This is the first report of quinolone resistance in nontyphoidal *Salmonella* isolated in the Mekong Delta in Vietnam, although Van *et al.* reported that *Salmonella* showing the resistance against enrofloxacin were isolated from retail raw food samples in Ho Chi Minh city in 2004 [32]. In addition, all the NA resistant isolates in the present study showed reduced susceptibility to CPFX. Hakanen *et al.* [11] suggested much lower MIC breakpoints values (MIC 0.25 μg/ml) for the fluoroquinolones than those recommended by the NCCLS (MIC 4 μg/ml) because of clinical importance of low-level CPFX resistance. If we adopt that breakpoint for the *Salmonella* isolates from the Mekong Delta in Vietnam, 4.8% (11 per 230) of the isolates should be considered resistant to ciprofloxacin. It is generally said that resistance to NA is a first-step of resistance to fluoroquinolones. Moreover, fluoroquinolones have been considered to be efficient antimicrobial agents against *Salmonella* infections and have been widely used. Therefore, as the emergence of quinolone resistance in *Salmonella* can be a serious public health problem, introduction of fluoroquinolones as food additives in food-producing animals, which would be a cause of inducing fluoroquinolone resistance to *Salmonella*,

is a cause for particular concern [10, 17, 35, 37, 38]. The results of this study, where quinolone resistant strains were detected in *Salmonella* isolates from animals and foods in Vietnam, suggested that fluoroquinolones are used in animal production as food additives or for treatment. Indeed, fluoroquinolones, such as norfloxacin and enrofloxacin, are sold at retail pharmacy for treatment of animal salmonelosis, but the detailed relationship among use of those antimicrobial agents and acquisition of quinolone resistance in the Mekong Delta is unclear due to the absence of reliable data on antimicrobial agents supplied to animals. However, the development of quinolone resistance, like in other countries, should also be considered.

Prudent use of antimicrobials in animal production system has been agreed worldwide to prevent development of antimicrobial resistance in pathogenic bacteria [5]. However, self-medication through retail pharmacies, which is a common practice in developing countries, is recognized as one of causes of inducing antimicrobial resistance in pathogenic bacteria. Similar to other developing countries, in Vietnam, antimicrobial agents can be bought easily in pharmacies without a prescription [5, 7]. Larsson *et al.* [16] also reported that ABPC, penicillin, amoxicillin, erythromycin, TC, and SM, respectively, are the most commonly used antimicrobial agents for treatment to acute respiratory tract infection in children in Bavi, Vietnam, and among the pathogens isolated from children, 88% of *Streptococcus pneumoniae* isolates and 32% of *Haemophilus influenzae* were resistant to TC, and 18% of *H. influenzae* and 19% of *Moraxella catarrhal* were resistant to ABPC. Considering that many antimicrobial agents for human and animals can be bought without control and therefore used inappropriately, increase of the antimicrobial resistance among *Salmonella* in Vietnam in future should be considered. Therefore, further investigation of *Salmonella* isolates from more extensive sources and continuous monitoring of antimicrobial resistance in Vietnam must be of great concern.

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Short Communication

Diagnosis and Assessment of Monkeypox Virus (MPXV) Infection by Quantitative PCR Assay: Differentiation of Congo Basin and West African MPXV Strains

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SUMMARY: Human monkeypox, an infectious disease caused by monkeypox virus (MPXV), is endemic to western and central Africa. A LightCycler quantitative PCR (LC-qPCR) system was developed for the diagnosis of this disease, targeting the A-type inclusion body gene (ATI gene) of MPXV. One naïve monkey was infected with MPXV Zr-599 (Congo Basin strain) and one with MPXV Liberia (West African strain). Another three monkeys were immunized with smallpox vaccine on 0, 3, or 7 days, respectively, before infection with MPXV Zr-599. Peripheral blood cell (PBC) and throat swab (TS) specimens were serially collected. The LC-qPCR was validated for the diagnosis of monkeypox using virus isolation. Sequencing of the partial ATI gene revealed the insertion of a unique 453-nucleotide residue in the West African strains but not in the Congo Basin strains. Specific reverse primers for Congo Basin and West African strains were designed based on the unique sequence insertion. The LC-qPCR detected the MPXV genome, but not those of the other orthopoxviruses tested nor the varicella-zoster virus. Both the sensitivity and specificity of the LC-qPCR were over 90% in comparison to virus isolation when TS specimens were tested. Fourteen of the 15 virus isolation-positive PBC specimens showed positive reactions in the assay. Further, most PBC specimens collected from symptomatic monkeys in the later stage of illness showed positive reactions in the assay but negative reaction in virus isolation. It was possible to differentiate between these two groups with the LC-qPCR. Thus, the newly developed LC-qPCR is a useful and reliable diagnostic tool for MPXV infection.

Monkeypox virus (MPXV) infection in humans, known as human monkeypox and having symptoms similar to those of smallpox, is endemic to the western and central regions of Africa (1,2). MPXV belongs to the Family *Poxviridae*, Genus *Orthopoxvirus*. A sporadic human monkeypox outbreak was reported in the United States in 2003, suggesting that the disease must be regarded as an important re-emerging infectious disease (3-7). MPXVs form two genetically distinct clades, Congo Basin and West African strains, which are prevalent in the central and western regions of Africa, respectively (7). It has been reported that human monkeypox in western Africa is less severe than that in central Africa (3). Therefore, a rapid diagnostic tool for MPXV infection with the ability to differentiate the two clades is desirable. In the present study, quantitative real-time polymerase chain reaction (PCR) for amplification of the MPXV genome and differentiation of the two clades was developed, and its efficacy in the diagnosis of monkeypox was examined.

MPXV strains, Zr-599, Congo-8, Sierra Leone, Orangutan, Copenhagen, Liberia, SEN-79, and clinical isolates (1996-97 isolates in DRC) recovered from patients with human monkeypox in the Democratic Republic of Congo in 1996 and 1997 were used (8). Ectromelia virus (strain Hampstead), camelpox virus (strain J1E3), cowpox virus (strain Brighton Red) and vaccinia virus (strain Lister) were also used. Cynomolgus monkeys were immunized with smallpox vaccine, LC16m8 (8,9). DNA of varicella-zoster virus (VZV) (strain Webster), whose infections (chickenpox and zoster) are important virus infections that need to be differentiated from monkeypox, was used.

Five male cynomolgus monkeys (*Macaca fascicularis*) weighing 3,080-4,500 g were used in the experiments. Three monkeys were immunized with a smallpox vaccine, LC16m8, and another 2 monkeys were mock-immunized. The 3 LC16m8-monkeys, Zr-LC-0, Zr-LC-3, and Zr-LC-7, were inoculated subcutaneously with the challenge virus (10^6 pfu, MPXV Zr-599) at either 0, 3, or 7 days post immunization, respectively. One of the mock-monkeys, Zr-Mock, was inoculated with MPXV Zr-599 and the other, Liberia-Mock, with MPXV Liberia at the same dose.

Before and after the MPXV challenge, 5 ml of total peripheral blood cells (PBC) was drawn and the surface of the throat of each monkey was swabbed with a cotton-tipped swab every 3-4 days. The swab was inoculated onto and mixed in 2 ml of MEM supplemented with 2%FBS (MEM-2FBS). The virus was isolated using Vero E6 cell-monolayers from buffy-coat fractions obtained from monkeys challenged with MPXV as reported previously (8). The throat swab (TS) specimens were centrifuged at 3,500 rpm for 5 min, and 1 ml of the supernatant fraction was inoculated onto Vero E6 cell monolayers seeded in a 25-cm^2-culture bottle for virus isolation.

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After incubation for 1 h, the cells were cultured in MEM-2FBS for 5 days. When a cytopathic effect (CPE) was observed in the cell culture, the CPE agent was confirmed as MPXV by indirect immunofluorescence assay. Plaque numbers were then counted after fixation of cells with a 10% formalin solution and staining with crystal violet solution.

The nucleotide sequence of the A-type inclusion body (ATI) gene of MPXV strains used in the present study was determined by the direct sequencing method. The partial ATI gene was amplified from the DNA of MPXV using primer set ATI-up-1 (5′-AATACAAGGAG GATCT-3′) and ATI-low-1 (5′-CTTAACTTTTTCTTTCTC-3′) (10). The PCR product was used as a template for direct sequencing.

DNA was isolated from 200 μl of PBC and TS specimens using a Viral Nucleic Acid purification kit™ (Roche Diagnostics, Mannheim, Germany). Purified DNAs were used as templates. The LightCycler real-time quantitative PCR (LC-qPCR) was developed using primers and probes that were designed based on the sequence of ATI gene in the MPXV genome (8). The sequences of the primers and probes were as follows: LC-forward primer, 5′-GAGATTAGCAGACT CCAA-3′; fluorescein (FC)-probe, 5′-GCAGTCGTTCAACT GTATTTCAAGATCTGAGAT-3′-Fluorescein; LCRed640 probe, 5′-LCRed640-CTAGATTGTAATCTCTGTAGCATT TCCACGGC-3′-phosphorylation; and reverse primers (Reverse primer 1: 5′-GATTCAATTTCCAGTTTGTAC-3′ and Reverse primer 2: 5′-TCTCTTTTCCATATCAGC-3′). The reverse primers, Reverse primer 1 and 2, were designed according to the specific nucleotide sequences in MPXV Congo Basin and West African strains, respectively. The LC-qPCR using the Reverse primer 1 and Reverse primer 2 were designated "LC-qPCR-C" and "LC-qPCR-W", respectively. Internal controls for the measurement of viral genome copy numbers of the MPXV Congo Basin and West African strains were pGEM-T-easy vectors (Promega, Madison, Wis., USA) carrying the ATI gene of MPXV Zr-599 and Liberia strains, respectively, and included in each assay. Amplification conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 57°C for 10 s, and 72°C for 6 s, and melting reaction.

PCR products, which were amplified from each of the MPXV strains by PCR using the primer set ATI-up-1 and ATI-low-1, were classified into 2 groups (1,520 bp and 1,070 bp, respectively, including the primer lesions) based on the size of the product (data not shown). An additional 453 nucleotide residues were present in the ATI gene of the Liberia, Copenhagen, SEN-79, Sierra Leone, Anteaten, and Orangutan MPXV strains at a point corresponding to position 2147/2148 of the nucleotide sequence of MPXV strain Gabon (one of the MPXV Congo Basin strains), counted from the initiation codon (GenBank accession no. MVU84504), resulting in a larger-size PCR product. On the other hand, Zr-599, Congo-8, and the 1996-97 isolates in DRC showed a smaller-size PCR product. These results indicate that the West African strains of MPXV have a longer ATI gene than the MPXV Congo Basin strains.

The LC-qPCR detected at least several copies of the MPXV ATI gene. Twenty-four PBC specimens and 24 TS specimens were serially collected from 4 monkeys on days 0-21 after infection with MPXV-Zr-599. Seven PBC specimens and 7 TS specimens were also serially collected from one monkey on days 0-21 after infection with MPXV-Liberia (Table 1). All the 18 virus-isolation-positive TS specimens showed a positive reaction in the LC-qPCR, while 12 of the 13 virus isolation-negative specimens showed a negative reaction.

Table 1. Relationship between the results of virus isolation and LC-qPCR among PBC- and TS-specimens

		LC-qPCR with					
		TS-specimens			PBC-specimens		
		+	−	Total	+	−	Total
Virus isolation	+	18	0	18	14	1	15
	−	1	12	13	7	9	16
Total		19	12	31	21	10	31

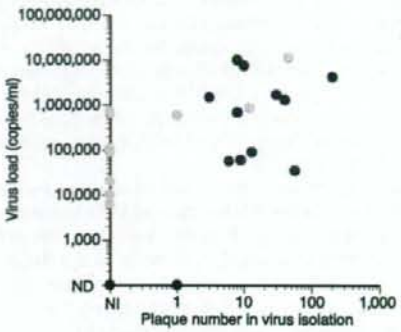

Fig. 1. Relationship between the virus load determined by the LC-qPCR and the plaque number in the virus isolation among PBC specimens. The specimens, which showed positive reactions in either virus-isolation or LC-qPCR, collected between day 0-10 and day 11-21 are shown in black circle and gray circle, respectively. The specimens, which showed positive reactions both in virus isolation and qLC-PCR are also shown in black circle. "ND" and "NI" indicate "undetectable" and "not isolated", respectively.

The sensitivity and specificity of the LC-qPCR were 100% (12/12) and 93% (12/13), respectively, in comparison with the virus isolation method. In contrast, 14 of the 15 virus isolation-positive PBC specimens showed a positive reaction in the LC-qPCR, while only 9 of the 16 virus isolation-negative specimens showed a negative reaction (Table 1). Most of the PBC specimens collected from symptomatic monkeys (Zr-Mock, Liberia-Mock, and Zr-LC-0) in the later stage of illness showed a negative reaction in virus-isolation negative but a positive reaction in the LC-qPCR (Fig. 1). IgG-response is usually demonstrated in monkeys subcutaneously infected with MPXV after approximately 10 days post inoculation (8). It is speculated that the discrepancy between the virus isolation and the LC-qPCR in PBC specimens in the later stage of illness is due to IgG-response. The antibody reactive to MPXV may make the virus isolation test negative, even though PMXV is represented in the PBC specimens.

The LC-qPCR-C detected only MPXV Congo Basin strains, whereas LC-qPCR-W detected only MPXV West African strains. The LC-qPCR detected MPXV DNA, but not the DNAs of camelpox virus, cowpox virus, ectromelia virus, vaccinia virus, or VZV, whereas the DNAs of these viruses were detected by conventional PCR methods using the primer set, ATI-up-1 and ATI-low-1, or the in-house primer set designed for the thymidine kinase gene of VZV (data not shown). There is a unique specific nucleotide sequence with a unique 8-nucleotide residue deletion only in the ATI gene of MPXV (11). An LCRed probe was designed to anneal this MPXV-specific nucleotide sequence. The specific reaction to MPXV in the LC-qPCR is thought to be due to the specific nucleotide sequences (11). The DNA of variola virus, the

causative agent for smallpox, expected to show a negative reaction in the LC-qPCR, as variola virus does not contain the specific 8-nucleotide residue deletion. The fact that the homology of the target region in MPXV with the corresponding region in the variola virus is lower than those with the corresponding regions in the orthopoxviruses tested supports this assumption.

We succeeded in producing various levels of clinical symptoms in 4 MPXV-infected monkeys. The naïve-monkey (Zr-Mock) died, while the Zr-LC-0 and Zr-LC3 monkeys survived. The ZR-LC-7 monkey did not show any symptoms. The viremia level determined by LC-qPCR in the Zr-Mock PBC continued to increase during the course of illness, while viremia was not demonstrated in the asymptomatic monkey, Zr-LC-7, by the assay. LC-qPCR is considered to be efficacious not only in diagnosis of MPXV infections but also in the assessment of the severity of MPXV infection-associated symptoms and outcome.

Recently, there have been several reports that describe real-time qPCR assays for the detection of MPXV genomes (12-16). These real-time qPCR assays, including that described herein, are sensitive, rapid, and useful in the diagnosis of MPXV infections. The significant advantage of this newly developed assay, LC-qPCR, over previously reported methods is that it enables the differentiation of MPXV into West African and Congo Basin strains.

In summary, we developed a sensitive, specific, and rapid LC-qPCR system for detection of the MPXV genome targeting specific nucleotide sequences in the ATI gene. This technology should offer great benefits in the control of outbreaks of MPXV infections and in the assessment of the course of MPXV infections.

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We thank Dr. N. Inoue for providing us with the DNA of varicella-zoster virus Webster strain.

The monkeys were born and raised in the Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Tsukuba, Japan (formally the Tsukuba Primate Center for Medical Science, NIID). The challenge experiments with MPXV were conducted in a highly containment laboratory at the NIID, Tokyo, Japan. The animal experiments were conducted with the approval from the Ethical Committee on Animal Experiments, NIID, Japan.

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Ligation-mediated amplification for effective rapid determination of viral RNA sequences (RDV)

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Abstract

Background: Emerging infectious diseases pose a significant risk to public health. Methods for rapid detection of pathogens are needed to effectively treat these diseases. Recently, we developed new methods for the rapid determination of viral RNA sequences, RDV ver1.0 and ver2.0. We demonstrated that these methods were able to simultaneously detect cDNA fragments of many different viruses without using sequence specific primers. However, some species of viruses, including the Yokose virus (YOKV), a flavivirus, could not be detected using the conventional procedures.

Objective: The RDV method was further modified to reduce the candidate PCR primer sets.

Study design: Primer sets were reduced to 256 sets in the improved RDV ver3.0, and theoretically, all viral cDNA fragments ligated by two kinds of adaptors after digestion by two restriction enzymes could be amplified in the PCR step for direct sequencing.

Results: We succeeded in obtaining 118 YOKV cDNA fragments of the 141 sequence fragments. The cDNA fragments covered diverse range of viral genome.

Conclusion: We were able to reduce the combinations of PCR primer sets used in the RDV method. This RDV method ver3.0 has a potential to detect viral cDNA fragments of both known and unknown RNA viruses rapidly and conveniently.

Keywords: RDV; Rapid determination; Direct sequence; Yokose virus; Virus discovery

1. Introduction

Recently, we developed a method for sequence-independent detection of RNA viruses, the "rapid determination of RNA virus (RDV) method" (Mizutani et al., 2007). In the RDV method, viral genome sequences are obtained without sub-cloning into plasmid vectors. Nucleic acid sequences of severe acute respiratory syndrome coronavirus, murine hepatitis virus, West Nile virus, Japanese encephalitis virus, and Dengue virus were successfully detected in culture supernatants from virus-infected cells by RDV ver1.0, which is our original method for detecting RNA viral genomes (Mizutani et al., 2007; Kihara et al., 2007). To increase sensitivity over RDV ver1.0, the sequence-independent amplification step was improved. By using the improved method, RDV ver2.0, avian paramyxovirus was detected in the allantoic fluid of embryonated chicken eggs (Sakai et al., 2007). A new adenovirus was successfully detected in the culture supernatant of primary kidney cells originating from a fruit bat using this RDV method (RDV-D) (Maeda et al., 2008). RDV methods have the potential to become standard methods for the

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detection of both known as well as newly emerging unknown viruses in humans and animals.

For direct sequencing after amplification of cDNAs in RDV ver1.0 and ver2.0, we use specially designed primer sets in which 6 nucleotides consisting of the CC (HaeIII-digested sequence) and four variable nucleotides are added to the 3′ end of the adapter sequence. Therefore, very large numbers of combinations of primer sets ($4^4 \times 4^4 = 65{,}536$ sets) are theoretically necessary to detect all the viral genomes in a sequence-independent manner (Mizutani et al., 2007). To avoid this level of complexity, we conventionally used the primer sets fixed the 5′ end of two nucleotides in the four variable nucleotide region to AG for the forward primer or GG for the reverse primer. However, we found that some viral species are difficult to detect using the limited combinations of primer sets. For example, the Yokose virus (YOKV) was isolated from a bat in Japan in 1971, and it was found to be an Entebbe bat virus, genus *Flavivirus*, in the family *Flaviviridae*. Although the full genome sequence has been published recently (AB114858), little is yet known about the characteristics of the virus (Tajima et al., 2005). We attempted to detect the YOKV sequence in the culture supernatant from virus-infected Vero cells using the procedure of RDV ver1.0 and ver2.0, but this failed. Therefore, in this study, we further improved the RDV method to detect more virus species, in particular by modifying the adaptor ligation step, to reduce the candidates of PCR primer sets for direct sequencing. This RDV ver3.0 was able to detect over 100 cDNA fragments of YOKV.

2. Methods

2.1. Cells and virus

Vero cells were maintained in Dulbecco's modified Eagle's medium with 5% fetal calf serum, penicillin, and streptomycin. The Oita-36 strain of YOKV was kindly provided by Dr. Tomohiko Takasaki (National Institute of Infectious Diseases of Japan). The virus was propagated in Vero cells. At 2 days post-infection, the infectious fluid was harvested. Cellular debris was removed by low-speed centrifugation ($2000 \times g$, 15 min, 4 °C) and the resulting supernatant was collected.

2.2. Design and scheme of RDV ver3.0

The RDV ver3.0 method includes the four procedures described below (Fig. 1).

2.2.1. RNA extraction

Viral RNA was extracted from the infectious supernatant containing $10^{5.5}$ $TCID_{50}$ of viruses by using a Total RNA isolation mini kit (Agilent Technology, USA) in accordance with the manufacture's instruction.

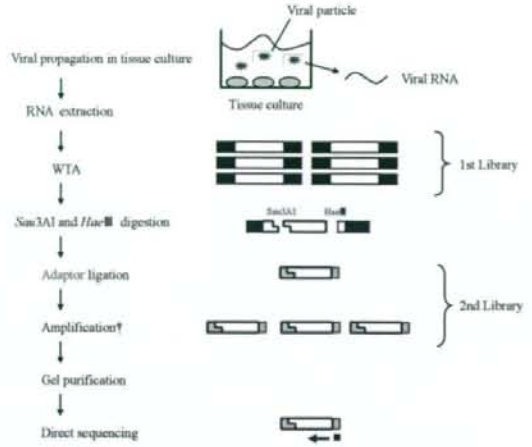

Fig. 1. Overall scheme for RDV ver3.0. WTA, whole transcriptome amplification; (†) with specially designed primer sets as shown in Fig. 2B.

2.2.2. Construction of first cDNA library

A whole transcriptome amplification system (WTA; Sigma–Aldrich, Saint Louis, MO, USA) was used to amplify viral double-stranded cDNA in accordance with the manufacture's instruction. PCR was performed as described in previous paper (Mizutani et al., 2007).

2.2.3. Second cDNA library

After the first cDNA library purification using the MonoFas DNA isolation system (GL Science, Japan), DNA was digested with 40 U of HaeIII (Takara Bio Inc., Japan) and Sau3AI (Takara Bio Inc.) at 37 °C for 30 min, and then the digested DNA was again purified using MonoFas. For construction of the second cDNA library, 2.5 μl of DNA solution, 2.5 μl of distilled water, 2.5 μl of sticky-ended adaptor, Adaptor-Sse83871 (10 μM) and blunt-ended adaptor, Adaptor-NotI (10 μM) were mixed (Fig. 2A). Ligation-convenience kit (Nippon Gene, Japan) was used for adaptor ligation. The DNA solution and 10 μl of ligation mix were reacted at 16 °C for 30 min, and the DNA was isolated using MonoFas. The second cDNA library was amplified by PCR using specially designed primer sets, and the forward primers in which six nucleotides included GATC (Sau3AI-digested sequence) and two variable nucleotides were added to the 3′ end of the Adaptor-Sse83871 sequence, and the reverse primers in which four nucleotides included CC (HaeIII-digested sequence) and two variable nucleotides were added to the 3′ end of the Adaptor-NotI sequence (Fig. 2B). PCR was performed as described in Sakai et al. (2007).

2.2.4. Direct sequencing

After electrophoresis of PCR products on agarose gels, the bands over 150 bp in length were excised, and DNA was extracted from the gel using the MonoFas. Direct sequencing was performed using the forward or reverse primer.

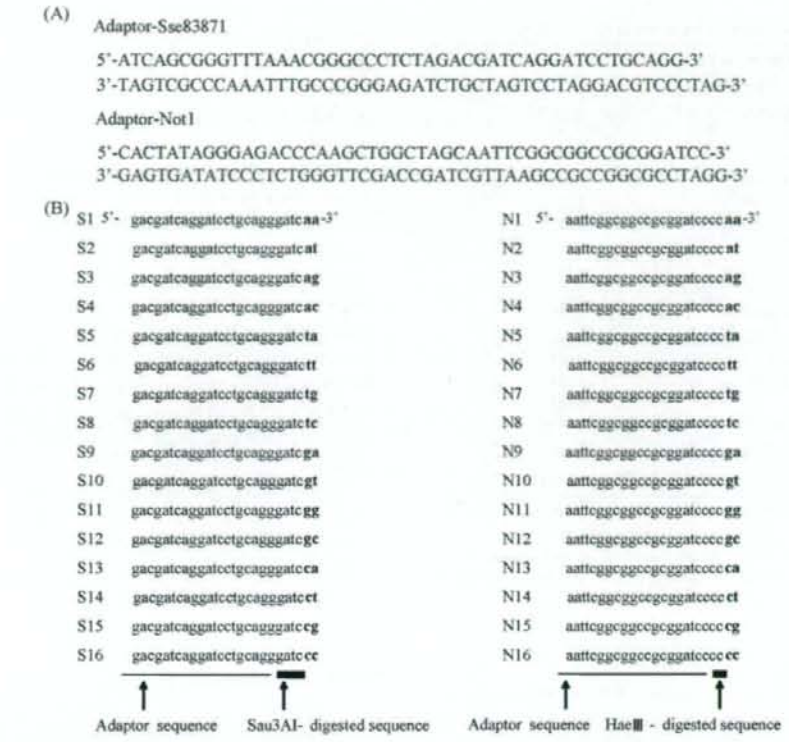

Fig. 2. The design of adaptors and primer sets used in RDV ver3.0. (A) Adaptor-Sse83871 contains sticky-end structures digested with Sau3AI, whereas Adaptor-NotI contains blunt-end structures digested with HaeIII. (B) All primers used in RDV ver3.0. Bold letters indicate 2-variable nucleotides.

3. Results

In RDV method ver3.0, different adaptors were ligated at the 5′ and 3′ end of viral dsDNA. The ligation enzyme was changed to the ligation mix in the Ligation-convenience kit (Nippon gene) because this increased the efficacy of ligation (data not shown). The S1–S16 forward primers and N1–N16 reverse primers were used in the second cDNA amplification step for direct sequencing (Fig. 2B). Using the RDV ver3.0, we successfully identified West Nile virus and Dengue virus type 2, which were used in previous our study (Mizutani et al., 2007) (data not shown). The RDV ver3.0 method was performed using extracted RNA from the culture supernatant obtained from YOKV-infected Vero cells. The 256 PCR

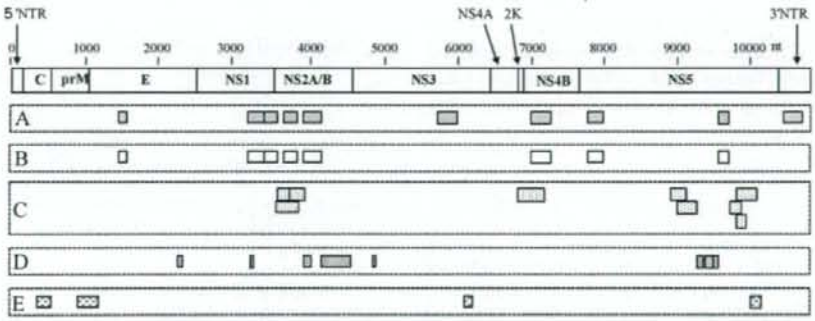

Fig. 3. Location of viral cDNA fragments on YOKV genome amplified using the RDV ver3.0. Column A shows 10 cDNA fragments expected to be detected in RDV ver3.0 (over 150 bp cDNA fegments digested by Sau3AI and HaeIII). Columns B–E show YOKV cDNA fragments detected in RDV ver3.0. Column B shows 8 of 10 cDNA fragments as shown in A. Column C shows cDNA fragments partially digested with HaeIII or Sau3AI. These cDNA fragments contained undigested sequences by the restriction enzymes. Column D shows amplicons detected as 2 or 3 ligated cDNA fragments. Column E shows cDNA fragments not containing recognition sequences of restriction enzymes at the ends.

Table 1
Number of sequence DNA fragments detected in RDV ver3.0

Forward primer	Number of sequence DNA fragment	Number of expected DNA fragment
S1	11/11 (100) [a]	2 (2) [b]
S2	21/21 (100)	2 (2)
S3	3/3 (100)	0 (0)
S4	5/8 (62.5)	0 (0)
S5	11/11 (100)	2 (2)
S6	18/18 (100)	1 (1)
S7	1/3 (33.3)	0 (1)
S8	8/11 (72.7)	0 (1)
S9	11/11 (100)	0 (0)
S10	11/16 (68.7)	0 (0)
S11	5/6 (83.3)	0 (0)
S12	3/6 (84.17)	0 (0)
S13	4/6 (66.6)	1 (1)
S14	0/0	0 (0)
S15	0/1 (0)	0 (0)
S16	5/12 (41.6)	0 (0)
Total	118/141 (83.6)	8 (10)

[a] Number of YOKV cDNA fragments/number of sequence DNA fragments (percent).
[b] Number of detected YOKV cDNA fragments of (number of YOKV cDNA fragments expected to be detected from complete nucleotide sequence).

products were processed by agarose gel electrophoresis. A total of 141 fragments were sequenced by the direct sequencing method, but sequences of 10 fragments were not obtained. One hundred eighteen exhibited high degrees of homology with the nucleic acid sequence of YOKV. Twenty-two DNA fragments were identified as mammal ribosomal RNAs (data not shown). The locations of the PCR fragments found in the YOKV genome are shown in Fig. 3. From the complete nucleotide sequences of YOKV, there are 10 cDNA fragments over 150 bp with HaeIII- and Sau3AI-digested ends (Fig. 3 column A), which are expected to be detected using RDV ver3.0, and eight fragments were detected (Table 1 and Fig. 3 column B). Nine were obtained as partial digested viral cDNA fragments by HaeIII or Sau3AI (Fig. 3 column C). In addition, we found two or three ligated viral cDNA fragments (Fig. 3 column D) and cDNA fragments not containing recognition sequences of restriction enzymes at the ends (Fig. 3 column E). The limit of detection of RDV ver3.0 was approximate 10^4 copies of viral genomic RNA as a starting material when using YOKV (data not shown).

4. Discussion

In this study, we succeeded in reducing the candidates of PCR primer sets for direct sequencing by improving the adaptor ligation step in RDV method. Theoretically, by using all combinations of the S1–S16 forward and N1–N16 reverse primers, all viral cDNA fragments ligated by two kinds of adaptors could be amplified in the direct sequencing step. Therefore, our newly developed RDV ver3.0 has been demonstrated to be superior in detecting unidentified viruses within at most 256 PCR reactions.

After direct sequencing, we found that viral DNA fragments could be amplified by PCR with a primer set in which one of two variable nucleotides in forward or reverse primers are matched to the target fragment. We obtained amplicons containing two or three ligated DNA fragments and also obtained viral DNA fragments partially digested with HaeIII or Sau3AI (Fig. 3). As shown in Fig. 3, many DNA fragments, covering a diverse range of viral nucleotide sequences, were detected. This suggests that the sequenced fragments detected by RDV ver3.0 can be used for the design of primers to determine complete viral nucleotide sequences for long PCR when unknown viruses are detected.

In this study, we were able to reduce the combinations of PCR primer sets used in the RDV method to a very large degree. This RDV method ver3.0 promises to greatly improve sequence-independent detection of RNA viruses especially when emerging virus disease occurs.

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Expert Opinion

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Real-time quantitative polymerase chain reaction for virus infection diagnostics

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Background: The development of real-time quantitative polymerase chain reaction (real-time qPCR) technology for the quantification and detection of the target genome with high sensitivity offers great advantages for the diagnosis of infectious diseases. This technique has become widely used for the diagnosis and assessment of viral infectious diseases. *Objectives*: The aim of this review is to describe the technologies of real-time qPCR that have been developed recently, applications of the real-time qPCR on virus infectious diseases, and to discuss the circumstances of real-time qPCR in the area of virus infectious diseases. *Conclusion*: The possibility of false-positive and false-negative results always exists when performing real-time qPCR. Reducing these risks is important. The use of real-time qPCR in the field of diagnostics for viral infections is likely to increase. The standard of performance of real-time qPCR in each institute needs to be maintained, thus requiring the establishment of a framework for the standardization of assay quality among the laboratories responsible for the diagnosis of infectious diseases.

Keywords: diagnosis, infectious diseases, quality assurance, real-time quantitative polymerase chain reaction, viral infections

Expert Opin. Med. Diagn. (2008) 2(11):1-17

1. Introduction

The characteristics of infectious diseases have changed dramatically in recent years in terms of etiology, treatment and prevention, and the interaction between infectious diseases and the community. Treatment strategies for viral diseases based on antiviral agents have been well developed for some virus infections such as herpesvirus, hepatitis B virus (HBV), hepatitis C virus (HCV) and human immunodeficiency virus (HIV) [1]. Newly developed vaccines have also become available for clinical use for several infectious diseases such as rotavirus diarrhea and cervical cancer-caused human papillomavirus [2,3]. There has also been an increase in the popularity of organ transplantation-based therapies thanks to the development of efficacious immune-suppressive agents. On the other hand, the number of patients with immunodeficiency has also increased. Virus infections in immunocompromised patients have become a major concern as the nature of infections in such patients differs from those in immunocompetent subjects in terms of severity, duration, morbidity and mortality. Several infectious diseases caused by new etiological agents have emerged in recent years, such as SARS, highly pathogenic avian influenza virus A (H5N1) infections and Nipah encephalitis [4-6]. Furthermore, it is well known that we now face a possible threat of bioterrorism in which variola virus or other dangerous pathogens could be used as bioweapons [7]. Therefore, the characteristics of the diagnostics used for infectious diseases have become one of the most important issues for the treatment and prevention of such infectious diseases.

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The diagnosis of infectious diseases is based on the detection of the causative agent by the isolation of pathogens, the detection of antigens, and the amplification of the virus genome and/or demonstration of a significant rise in the antibody titer to the causative agents between acute and convalescent phases.

Based on the circumstances described above, precise and rapid diagnosis of infectious diseases is inevitably necessary. The diagnostic methods for infectious diseases were revolutionized by the introduction of polymerase chain reaction (PCR), which was developed ~ 20 years ago. Various molecular techniques have been developed since the introduction of PCR, and these technologies have matured sufficiently to make them viable for use as diagnostic tools in recent years. The development of real-time and quantitative PCR, including real-time reverse-transcription PCR (real-time qPCR), is foremost owing to its sensitivity in the detection and quantification of the pathogen genome in samples. In this regard, real-time qPCR is considered to be useful not only for the detection of etiological agents, but also for the assessment of infectious diseases in patients.

In this review, real-time qPCR in diagnosis of virus infections in humans is introduced and assessed. The advantages and disadvantages of real-time qPCR over other diagnostic methods are also discussed. Furthermore, the importance of maintaining high standards with regard to the performance of real-time qPCR will be emphasized.

2. Technologies for real-time qPCR

Real-time qPCR technology is based on the detection and quantification of a fluorescent reporter [8,9], which increases in accordance with the amount of PCR product in the reaction. The higher the copy number of the target genome, the sooner a significant increase in fluorescence is observed. Two systems for the real-time qPCR have been developed: real-time qPCR, in which the nonspecific DNA intercalating dyes SYBR® green [10] or Syto9 [11] are used, and those in which sequence-specific oligoprobes conjugated with a donor and acceptor fluorophor are used [12]. The sequence-specific oligoprobes carry a donor and acceptor fluorophor and use fluorescent resonance energy transfer (FRET) [12]. Two fluorophores, a donor and an acceptor, are required in FRET. Excitation of the donor by an energy source such as a fluorometer laser triggers an energy transfer to the acceptor if they are within a given proximity to each other. The acceptor in turn emits light at the given wavelength. In the former system using nonspecific DNA intercalating dyes, the dyes are incorporated into the amplification products. The fluorescent signals detected are not always specific, requiring confirmation of whether the signals are specific or not by denaturation (melting) curve analysis at the completion of the reaction. In the latter system, which uses sequence-specific oligoprobes, FRET hybridization probes anneal with the target nucleotide sequence.

The fluorescent signals detected, therefore, are probably due to increased PCR products from the specific pathogen genome. Two methods for this sequence-specific oligoprobe-based real-time qPCR have been developed: the single-labeled probe system; and the dual-labeled hybridization probe system. The single-labeled hybridization probe system is widely used in conjunction with the LightCycler instrument (Roche Diagnostics, Basel, Switzerland) [13]. The single-labeled probe system, in which two single-labeled oligoprobes, also referred to as LightCycler hybridization probes, are required, is widely used in the real-time qPCR. The two probes are the DNA probes designed to anneal nest to each other in a head-to-tail configuration on the PCR product. The upstream probe is labeled with a fluorescent dye of the 3′ end and the downstream probe is labeled with an acceptor dye on the 5′ end. The 3′ end of the downstream probe must be phosphorylated to prevent it being used as a prime by *Taq* polymerase during PCR amplification. If both probes anneal to the PCR product, fluorescent energy from the 3′ dye is absorbed by the adjacent acceptor dye on the 5′ end of the downstream probe. The acceptor dye is excited and emits light at a given wavelength and the light signal is detected. Two fluorescently labeled oligonucleotides are also used in the dual-labeled hybridization system; a fluorescent dye-labeled donor probe at the 3′ end and an acceptor probe labeled at the 5′ end, which absorbs resonance energy from the donor probe through FRET. Fluorescence from the acceptor probe is induced only when both the donor probe and the acceptor probe have annealed to the amplification product in close proximity to each other. Melting curve analysis of the amplification products makes it possible to confirm PCR products as the authentic amplification product, as the melting curve pattern is dependent on sequence variation in the probe target sites. The dual-labeled probe system refers to real-time qPCR using TaqMan probes [14], molecular beacons [15] and scorpion probes [16]. Real-time qPCR with TaqMan probes was the first to be developed and widely used. The TaqMan probes are oligonucleotides that contain a fluorescent dye and a quenching dye at the 5′-base and 3′-base, respectively, and are designed to anneal to a complementary sequence on a PCR product. The close proximity of the reporter and quencher prevents emission of any fluorescence while the probe is intact. The polymerase amplifies PCR products on which a TaqMan probe is bound. The 5′ exonuclease activity of the polymerase hydrolyses the probe and separates the quencher dyes from the fluorescent dye. This results in the emission of fluorescence, which increases in each cycle proportional to the rate of probe cleavage [17]. By monitoring the intensity of the fluorescence emission of the reporter dye, it is possible to quantify the level of pathogen genome in the sample. The viral load can be expressed in absolute values with reference to quantified standards, or in relative values compared with another target sequence present in the sample.

The isothermal amplification method for nucleic acids, NASBA, is a technology with the potential for broad applications in the field of RNA amplification and detection. A NASBA reaction is based on the concurrent activity of AMV reverse transcriptase (RT), RNase H and T7 RNA polymerase, together with two primers to produce amplification [18]. An introduction of a molecular beacon, which is designed to hybridize within the target sequence, in NASBA reactions that amplify the target RNA made it possible to quantify the amplified RNA genomes in real time [19]. NASBA possesses the ability to amplify homogenously and isothermally RNA analytes such as viral RNA and is applied as a method for the diagnosis of some RNA virus infections such as HIV and hepatitis C virus infections [20-25].

Recently, a new technology to amplify nucleic acid in a real-time manner, loop-mediated isothermal amplification (LAMP), has been developed [26]. LAMP is a nucleic acid amplification method that depends on the autocycling strand displacement DNA synthesis performed by *Bst* DNA polymerase. The amplification is conducted under isothermal conditions ranging from 60 to 65°C with DNA polymerase and usually 4 or 6 primers recognizing 6 distinct target regions, making this assay highly specific. Even the visual detection of the turbidity, derived from the accumulation of by-product, is possible [8]. Recently, the LAMP-based diagnostics have been developed for several virus infections, such as herpesviruses (HSV-1, HSV-2), CMV, EBV, respiratory viral pathogens, measles virus, rubella virus, mumps virus, hepatitis viruses, filovirus and flaviviruses [27-49].

3. Application of real-time qPCR for the diagnosis of infectious diseases

3.1 Advantages in the measurement of virus load by real-time qPCR

The real-time qPCRs developed for the diagnosis of virus diseases are summarized in **Table 1**. The advantages of real-time qPCR over conventional PCR and enzyme immunoassay-based viral antigen detection are 'quantification-ability', 'high sensitivity' and 'rapid assay'. Rapid diagnosis and quantification-ability are particularly important in the treatment and assessment of life-threatening infections such as respiratory virus infections [5,6], central nervous infections due to herpes simplex virus (HSV) [50,51], human herpes virus type 6 (HHV-6) [52,53] and JC virus (JCV) [54], infections in immunocompromised patients as described below. The ability of real-time qPCR to quantify the viral load in clinical specimens has made a major contribution to the treatment and assessment of infectious diseases in patients. So far, it has become possible for patients with several virus diseases, such as HSV, cytomegalovirus (CMV), HIV, HBV, HCV and influenza virus infections, to be treated with antiviral agents [1]. The measurement of viral load in samples, particularly in peripheral blood and/or cerebrospinal fluid (CSF), makes it possible to determine the dynamics of viral proliferation in patients. Assessment of the dynamics of viral load by real-time qPCR offers great advantages through the ability to monitor the efficacy of antiviral therapy, as it has been reported that the higher the viral load, the higher the severity of the disease [55-58]. Real-time qPCR is the most powerful tool for the detection of viruses that cannot be isolated using cell-culture techniques or that require a long time for isolation and identification. Such DNA and RNA viruses include JCV, BK virus (BKV), HBV and HCV, and norovirus.

3.2 Application of real-time qPCR to CNS infections

Real-time qPCR has been applied for the diagnosis of central nervous system (CNS) infections caused by viruses such as HSV-1, HSV-2 [59,60], varicella-zoster virus (VZV) [51], CMV [61], HHV-6 [62,63], JCV [58,64], enterovirus [65,66], measles virus [67], mumps virus [68], tick-borne encephalitis virus [69], Japanese encephalitis virus [70,71], West Nile virus [72], Toscana virus [73] and La Crosse virus [74]. Encephalitis caused by HSV-1, HSV-2 and VZV is usually life-threatening, necessitating differential diagnosis between these three viruses and between them and CNS infections caused by other pathogens. The diseases are treatable with an efficacious antiviral agent, acyclovir. Furthermore, virus isolation-based diagnostics for the disease are usually difficult and PCR has been recognized as the reference standard assay. The sensitivity and specificity of real-time qPCR were both > 90% in comparison with conventional nested PCR assay [51]. It is considered that the conventional PCR will be superseded by real-time qPCR owing to the high sensitivity and specificity, rapidity, and quantitative performance of the latter method. It is noteworthy that real-time qPCR is an alternative to conventional PCR for the diagnosis of neonatal infections associated with these viruses as well because HSV and VZV infections in neonates cause severe and generalized infections with a high mortality rate [50]. JCV is the causative agent for progressive multifocal encephalopathy (PML) [75]. PML usually occurs in severely immunocompromised patients and is life-threatening. The isolation of JCV remains impossible, therefore molecular detection by PCR has long been recognized as a sensitive and specific method for the detection of JCV in clinical samples. Although PML infections are not treatable with an antiviral agent, measuring the viral load in CSF would provide useful information on the treatment strategy [58]. Real-time qPCR seems certain to become the standard assay for the diagnosis of PML in the near future.

3.3 Application of real-time qPCR to respiratory tract infections

The common etiological viruses for respiratory tract infections identified so far are influenza virus, respiratory syncytial virus (RSV), parainfluenza viruses, human metapneumovirus, rhinoviruses, human coronaviruses, adenoviruses, enteroviruses and the newly identified human bocavirus. However, > 50% of cases remain without an etiological diagnosis. Cell

Table 1. Application of real-time qPCR to typical infections caused by viral agents.

Infection type	Agents	Genome type	Diseases	Sample type	Type of system	Target gene	Ref.
CNS infections	HSV-1	DNA	Encephalitis and meningitis	CSF	TaqMan	DNA polymerase (*UL30*)	[50]
						gD gene	[51,61]
						gG gene	[60]
					SYBR Green I	*gD* gene	[59]
	HSV-2				TaqMan	DNA polymerase (*UL30*)	[50]
						gD	[51,60]
					SYBR Green I	*gG*	[59]
	VZV				TaqMan	DNA polymerase	[51]
					SYBR Green	*ORF 29* gene	[59]
	CMV				TaqMan	*MIEA* gene	[61]
	HHV-6				TaqMan	*U6* gene	[63]
	EBV				TaqMan	*EBNA-1*	[61]
	JCV		PML		TaqMan	*VP2* gene	[54]
					TaqMan	Large T-antigen	[58,64]
	EV	RNA	Meningitis and encephalitis		TaqMan	5′-non coding region	[65]
					SYBR Green I	5′-non coding region	[66]
	MV		Meningitis and encephalitis, SSPE		SYBR Green I	N, M and H protein gene	[67]
	Mumps virus		Meningitis and encephalitis		TaqMan	*SH* gene	[68]
	JEV				TaqMan	*NS3* gene	[70]
					SYBR Green I	*NS3* gene	[71]
	WNV				TaqMan	5′-untranscribed region/protein C gene	[72]
	Toscana virus				TaqMan	S-segment	[73]
	La Crosse virus				TaqMan	M-segment	[74]
	TBEV				TaqMan	3′-non coding region	[69]

ADV: Adenovirus; B19: Human parvovirus B19; CHIKV: Chikungunya virus; CMV: Cytomegalovirus; CSF: Cerebrospinal fluid; DV: Dengue virus; EBOV: Ebolavirus; Flu A: Influenza virus A; Flu B: Influenza B; GTI: Gastrointestinal tract infections; HPV: Human papillomavirus; HSV: Herpes simplex virus; HHV-6: Human herpes virus type 6; JCV: JC virus; JEV: Japanese encephalitis virus; LC: LightCycler™; MBRV: Marburgvirus; MPXV: Monkeypox virus; NoV: Norovirus; NPA: Nasopharyngeal aspirate; PIV: Parainfluenza virus; RSV: Respiratory syncytial virus; RTI: Respiratory tract infection; RV: Rotavirus; SaV: Sapovirus; SSPE: Subacute sclerosing panencephalitis; TEBV: Tick-borne encephalitis virus; VHF: Viral hemorrhagic fever; WNV: West Nile virus.

Table 1. Application of real-time qPCR to typical infections caused by viral agents (continued).

Infection type	Agents	Genome type	Diseases	Sample type	Type of system	Target gene	Ref.
RTI	ADV	DNA	Upper and lower RTI	NPA/peripheral blood	TaqMan	*Hexon* gene	[83]
					SYBR green I	*Hexon* gene	[84]
	RSV	RNA		NPA	TaqMan	*N* and *F* genes	[77,78]
						N gene	[77,78]
						M2 gene	[81]
					Single probe (LC)	*N* gene	[80]
					SYBR Green I	*F* protein gene	[81]
	PIV				TaqMan	Hemagglutinin-neuraminidase gene	[79]
	hMPV				TaqMan	*F* gene	[82]
	Flu A				TaqMan	*Matrix* gene	[76]
						NS2 gene	[81]
					SYBR Green I	*Matrix* gene	[79]
	Flu B				TaqMan	*HA* gene	[76]
						NP gene	[81]
					SYBR Green I	*HA* gene	[79]

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Table 1. Application of real-time qPCR to typical infections caused by viral agents (continued).

Infection type	Agents	Genome type	Diseases	Sample type	Type of system	Target gene	Ref.
GTI	ADV F	DNA	Diarrheal disease	Feces	TaqMan	*Hexon* gene	[86]
	RV A	RNA			TaqMan	*VP6* gene	[86]
						VP3 gene	[87]
					SYBR green I	*VP6* gene	[88]
	RV C				TaqMan	*VP7* gene	[86]
	Astrovirus				TaqMan	Capsid protein precursor gene	[89]
	NoV G-I				TaqMan	RNA polymerase gene	[89]
						ORF1–ORF2 junction region	[90]
					SYBR Green I	Polymerase gene	[91]
	NoV G-II				TaqMan	*ORF1–ORF2* junction region	[90]
					SYBR Green I	*Capsid* gene	[91]
	NoV G-II and G-IV				TaqMan	RNA polymerase gene	[89]
	SaV				TaqMan	Polyprotein gene	[89]
Hepatitis	HBV	DNA	Hepatitis, cirrhosis, HCC	Peripheral blood, liver tissue	TaqMan	*HBV* surface gene	[94]
						X gene	[94]
						Core region gene	[97]
	HCV	RNA			TaqMan	5′-non coding region	[92]
					SYBR green I	5′-non coding region	[98]

ADV: Adenovirus; B19: Human parvovirus B19; CHIKV: Chikungunya virus; CMV: Cytomegalovirus; CSF: Cerebrospinal fluid; DV: Dengue virus; EBOV: Ebolavirus; Flu A: Influenza virus A; Flu B: Influenza B; GTI: Gastrointestinal tract infections; HPV: Human papillomavirus; HSV: Herpes simplex virus; HHV-6: Human herpes virus type 6; JCV: JC virus; JEV: Japanese encephalitis virus; LC: LightCycler™; MBRV: Marburgvirus; MPXV: Monkeypox virus; NoV: Norovirus; NPA: Nasopharyngeal aspirate; PIV: Parainfluenza virus; RSV: Respiratory syncytial virus; RTI: Respiratory tract infection; RV: Rotavirus; SaV: Sapovirus; SSPE: Subacute sclerosing panencephalitis; TEBV: Tick-borne encephalitis virus; VHF: Viral hemorrhagic fever; WNV: West Nile virus.

Table 1. Application of real-time qPCR to typical infections caused by viral agents (continued).

Infection type	Agents	Genome type	Diseases	Sample type	Type of system	Target gene	Ref.
Emerging and re-emerging virus infections	MPXV	DNA	Human monkeypox	Peripheral blood, vesicular fluid	Single probe (LC)	*ATI* gene	[114]
					TaqMan	DNA polymerase and *B6R* genes	[115]
	SARS-CoV	RNA	SARS	Peripheral blood, feces, tracheal aspirate	TaqMan	Not indicated	[104]
	Sudan EBOV		VHF	Peripheral blood	TaqMan	*NP* gene	[105]
	MARV				TaqMan	*VP40* gene	[100]
	Nipah virus		Encephalitis	CSF	TaqMan	*NP* gene	[99]
	MPXV		Human monkeypox	Peripheral blood, vesicular fluid	Single probe (LC)	*ATI* gene	[114]
					TaqMan	DNA polymerase and *B6R* genes	[115]
	Flu A (H5N1)		H5N1 flu infection	Tracheal aspirate	TaqMan	*HA* (*H5*) gene	[76,102,103]
	DV		Dengue fever/Dengue hemorrhagic fever	Peripheral blood	TaqMan	*E* gene	[108]
	CHIKV		Generalized infections	Peripheral blood	Single probe (LC)	*NSP1*	[110]
					SYBR green I	*E1* gene	[112]
Congenital virus infections	CMV	DNA	Congenital CMV infections	Amniotic fluid	TaqMan	*UL 123 exon 4* gene	[138]
				Blood spot	TaqMan	*UL 123 exon 4* gene	[121]
				Urine spot	TaqMan	*UL83* open reading frame	[122]
	B19		Congenital B19 infection	Peripheral blood, serum amniotic fluid, fetal ascites	TaqMan	*NS1* and *VP2* genes	[119]
	Rubella virus	RNA	Congenital rubella syndrome	Peripheral blood, serum amniotic fluid, fetal ascites	TaqMan	Non-coding region	[120]
Cervical cancer	HPV	DNA	Cervical cancer	Plasma	TaqMan	*E7* gene of HPV-16 and HPV-18	[139]
				Cytobrush specimens	SYBR green I	*E2* and *E6* genes of HPV-16	[140]
					TaqMan	*E7* transcript (RNA)	[118]
					SYBR green I	*E7* transcript (RNA)	[141]

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culture-based virus isolation techniques are reliable but remain costly and impractical for use in a clinical setting. Among the respiratory tract infections caused by the above-mentioned viruses, the rapid detection of the virus antigen using enzyme immunoassay and enzyme chromatography is available for the bed-side diagnosis of RSV and influenza viruses. Rapid and accurate diagnosis of respiratory tract infections is required for proper treatment of patients, especially for the proper prescription of antiviral agents, prevention of nosocomial infection, and collection of accurate epidemiological information. Real-time qPCR, which affords rapid and accurate diagnosis, has been developed for respiratory tract infections caused by the above-mentioned viruses as either a single virus-targeted real-time qPCR or multi-virus targeted real-time qPCR (multiplex real-time PCR) [76-84].

3.4 Application of real-time qPCR to gastrointestinal tract infections

Rotavirus, norovirus and enteric adenoviruses (serotypes 40 and 41) are the most common causative viral agents for gastrointestinal (GI) tract infections, and real-time qPCR (RT-PCR) has been developed for these virus GI tract infections. These viruses are so contagious that the possibility of nosocomial infection is a great concern, particularly in pediatric wards during the epidemic season. Although the mortality rate in children with rotavirus infections has recently fallen in developed countries, the morbidity of rotavirus and norovirus infections remains quite high. A rotavirus vaccine has become available in recent years [2,85]; however, rapid and accurate diagnosis is necessary for the proper treatment and assessment of GI tract infections in patients and a reduction in the risk of nosocomial transmission. Real-time qPCR assay offers great advantages in the pursuit of both these aims [86-91].

3.5 Application of real-time qPCR to hepatitis

HBV and HCV cause chronic infections and are the main causes of hepatitis and subsequent liver cirrhosis and hepatocellular carcinoma (HCC). In recent years, it has become possible to treat these infections with several antiviral agents. Based on the poor prognosis in some cases and the need to assess treatment efficacy, accurate diagnosis together with quantification of the viral load is required. HBV DNA or HCV RNA quantification is used to establish the prognosis for chronic HBV-related liver disease. For a proper assessment of the efficacy of treatment using antiviral agents, monitoring of the response and/or resistance to antiviral therapies is needed. Therefore, real-time qPCR-based assays are gradually replacing other technologies for the routine quantification of HBV DNA or HCV RNA in clinical practice [92-98]. Thus far, some real-time PCR tests for HBV and HCV infections have become commercially available. As real-time PCR can detect very low virus genome copy numbers, it offers significant advantages in the treatment and assessment of patients with HBV and/or HCV infections.

3.6 Application of real-time qPCR to emerging virus infections

We have been faced with the challenge of several emerging infections in the last decade. The viral hemorrhagic fevers caused by Ebolavirus and Marburgvirus have re-emerged in Africa on a scale greater than that previously observed. These infections are zoonotic and have a very high mortality rate. Nipah virus, Ebolavirus and Marburgvirus are internationally classified as biosafety level-4 pathogens. Therefore, the monitoring of these infections is an important step in the prevention of outbreaks. Real-time qPCR-based viral genome amplification systems have been developed for these emerging virus infections [99-105]. It is noteworthy that most of the emerging virus infections recently identified are caused by RNA viruses with a relatively high diversity in nucleotide sequence, suggesting that performances of the real-time qPCR should be repeatedly validated to ensure further improvement. In the cases of the Ebola hemorrhagic fever outbreak in Sudan in 2000 and the Marburg hemorrhagic fever outbreak in Angola in 2005, specific real-time qPCR was immediately developed and applied after the determination of the genome-nucleotide sequence of the viruses responsible for these outbreaks [100,105]. Although, the overall mortality rate is not so high, Dengue virus infections (mosquito-borne viral infections) are endemic to the tropical regions and sometimes result in a severe form of infection, such as dengue hemorrhagic fever or dengue shock syndrome. Furthermore, large-scale outbreaks of Chikungunya virus infections have re-emerged in Africa, islands in the Indian Ocean, and Southeast Asia in 2005 and 2006 [106]. A sporadic outbreak of Chikungunya virus also occurred in Italy for the first time through the importation of a Chikungunya virus-infected patient [107]. A real-time qPCR system was recently developed for these mosquito-borne re-emerging virus infections [108-112]. Monkeypox virus infections in humans, a disease similar to smallpox and known as human monkeypox, are endemic to central and western Africa. A sporadic outbreak of human monkeypox occurred in the US in 2003 [113]. Monkeypox virus is classified as a potential bioterrorism agent and a real-time qPCR system for this infection has been developed [114,115].

3.7 Application of real-time qPCR for papillomavirus infections responsible for cervical cancer

Cervical cancer is the leading cause of cancer morbidity in women. Human papilloma virus (HPV) is present in ~ 90% of high-grade dysplasias and cervical cancer [116]. Certain types of HPV play a pivotal role in the carcinogenesis of cervical cancer. Moreover, the amount of HPV type 16 DNA increased by orders of magnitude with increasing disease grade [117]. Measurement of HPV gene *E7* transcripts in cervical cells by real-time qPCR enables one to distinguish samples from patients with cervical cancer from those from subjects without cervical cancer [118]. As it is still impossible to isolate HPV, measurement of HPV load, especially the

virus load of HPV type 16, in samples such as cytobrush specimens, is useful for diagnosis of cervical cancer and for prediction of the outcome of the patient.

3.8 Application of real-time qPCR for the diagnosis of congenital virus infections

Rubella virus and CMV are leading causative virus agents for congenital infections. Although the number of patients with congenital rubella virus infection (congenital rubella syndrome) decreases according to the increase in rubella vaccination coverage in developed countries, congenital rubella syndrome is still one of the most important infections to be monitored. Congenital CMV infections in a fetus infected with CMV through a transplacental route when the mother was primary infected with CMV I at an early phase of pregnancy affects the developing neurological system, leading to sensorineural hearing loss and other neurological sequelae. Human parvovirus B19, a causative agent for benign erythema infectiosum, is one of the most important virus agents for congenital infections. Infection of the fetus with parvovirus B19 through a transplacental route causes congenital anemia, hydrops fetalis, and fetal death in severe cases. Diagnosis of these infections in pregnant subjects or of these infections in the fetus is required in some instances. For the diagnosis of such purposes, real-time qPCR has recently been applied because of a high sensitivity in detection of human parvovirus B19 nucleic acid from samples such as peripheral total blood [119]. The real-time qPCR diagnostics for CMV, human parvovirus B18 and rubella virus infections can be applied for congenital infections with these viruses [120]. Recently, real-time qPCR-based diagnostics have been applied for the screening of congenital CMV infections using dried blood spots on perinatal cards [121] or urine spots [122].

3.9 Application of real-time qPCR for the diagnosis of viral infections in immunocompromised patients

Infections in immunocompromised subjects are life-threatening in many cases. For example, HSV-1 infections in immunocompromised patients are generally more severe than those in immunocompetent subjects. CMV causes generalized infections such as pneumonia, retinitis, enteritis and encephalitis through the reactivation of latently infected CMV, whereas CMV does not cause such infections in immunocompetent subjects. Infections caused by HSV-1, HSV-2, VZV and CMV are now treatable with some antiviral agents, such as acyclovir, ganciclovir and foscarnet. However, drug-resistant infections have been reported in immunocompromised patients administered with the antiviral agents for longer periods of time. HHV-6 also causes encephalitis in immunocompromised patients, such as those receiving bone marrow transplantation [52,53]. MPL caused by JCV is also an important opportunistic infection. Latent infection with JCV causes PML in severely immunocompromised patients. BK virus, a human polyomavirus, also causes severe hemorrhagic cystitis in severely immunocompromised patients. Respiratory tract viral infections in immunocompromised patients are more severe and are sometimes life-threatening [123]. Accurate and rapid diagnosis together with the capacity to quantify viral load is required for the proper treatment and assessment of such patients [54,58,61,62,64,124-131]. Several real-time qPCR assays are commercially available for CMV infections. Pre-emptive monitoring for these infections with real-time qPCR offers the practitioners important information on the treatment of patients with immunodeficiency. It may make it possible to modify the treatment strategies, to prescribe the effective antimicrobial agents, and to assess the course of infections.

4. Practical issues regarding real-time qPCR quality control

4.1 Extraction of nucleic acids

Viral nucleic acids (double-stranded DNA, single-stranded DNA, double-stranded RNA and single-stranded RNA) should be properly extracted for real-time qPCR assays because this step is as critical as any steps in the real-time qPCR. The purification efficiency, inhibitor-removing efficiency, and reducing the risk of cross-contamination are required in the process of viral nucleic acid purification. Recently, various commercially available methods have been used for the extraction of viral nucleic acids from a wide variety of specimens. The commercially available kits for the extraction of viral nucleic acid, which are relatively widely used, are shown in **Table 2**. Viral nucleic acids are not extracted from samples by phenol/chloroform extraction methods. The specimen throughput is < 1 h when the maximum number of samples is manipulated at once. Furthermore, some manufactures such as Roche [132], Qiagen [133], Applied Biosystems [134] and bioMerieux [135] manufacture automated extraction instruments. The advantages of the viral nucleic acid extraction by using automated extraction instruments are as follows. Recovery of nucleic acids is consistent and reproducible. The risk of cross-contamination of samples is minimized because many of the instruments are closed system. The introduction of automated extraction systems should be considered in laboratories in which many clinical samples must be manipulated.

4.2 Minimization of the risk of false-positive results

False-positive and false-negative results should, obviously, be avoided. The efficiency of genome amplification by real-time qPCR is 100 – 1000 times more sensitive than that by conventional PCR and comparable with that of nested PCR. This very high sensitivity requires the careful management of the real-time qPCR procedure to avoid or minimize the risk of false-positive results resulting from contamination. Adequate positive and negative controls must be included as well as standards and samples in each assay batch to confirm quality and test performance. The positive and negative