Table 4. Detected regions in HCoV-HKU1 genome.

Region (nt)	Genome
3,854–4,083	Orf 1ab (Replicase)
15,956–16,147	Orf 1ab (Replicase)
24,506–24,653	Spike glycoprotein
28,082–28,310	Membrane glycoprotein - Nucleocapsid phosphoprotein

Reference sequence: HCoV HKU1 strain N15 genotype B, complete genome (NCBI accession number: DQ415911).
doi:10.1371/journal.pone.0004219.t004

the primers used for H1N1 and H3N2 influenza viruses (Table 1) or to the presence of different amounts of host-derived DNA/cDNA. Thus, quantitative analysis of host genes will be required. One potential reason for why we obtained fewer Flu-specific reads than norovirus reads in this study might have been the large number (90.0–94.6% of all reads) of host-derived sequences (Figure 2 and Table 5). These sequences were present because we performed direct RNA isolation from nasopharyngeal aspirates without first eliminating the cells or tissues. Most of the detected human-derived reads were non-coding regions, and fewer coding regions, including rRNA and mRNA sequences, were detected than expected (Table 5). These results suggest that, although contamination by human genomic DNA might be very low, an additional step for host gene removal is required. Suitable subtraction step(s) using pooled human genomic DNAs as drivers might be required to enrich in microbial genomes [15]. Alternatively, MICROBEnrich (Ambion Inc.), another method for removing contaminated human-derived RNA, could be useful to enrich microbial RNA [33]. However, the DNA virus WUV was detected from the isolated RNA, suggesting that the WUV genome and/or its transcripts present in infected cells were detected. Indeed, a novel human polyomavirus (Merkel cell polyomavirus), isolated from skin carcinoma, was detected from mRNA [17]. Taken together, these results indicate that whole RNA isolation, including host cells and tissues, followed by the suitable elimination of host-derived genes could be an effective method for identifying pathogenic viruses in clinical samples.

When several pathogens are found in a single sample, a careful interpretation is necessary to decide which pathogen(s) is the real cause of a specific disease. Although, the most abundant pathogen might generally be considered to be the best candidate,

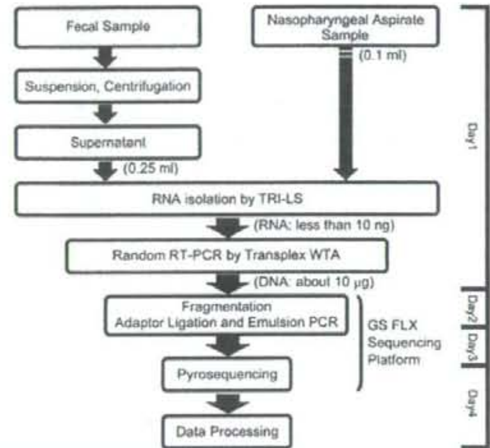

Figure 4. Process diagram for the viral diagnosis of nasopharyngeal aspirates and fecal samples.
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cooperative interactions between multiple pathogens cannot be excluded as an important factor for pathogenesis. To address this question, suitable control samples from healthy persons and/or pair specimens, isolated after recovery, might be required.

Another possible problem with this viral genome analysis is biased cDNA synthesis by quasi-random RT-PCR with the WTA kit. As shown in Figure S1, a significant bias was found and its pattern was identical in all samples. TG (CA)–rich regions were selectively amplified with the WTA kit (Table S3), probably due to nucleotide sequences of the quasi-random primer. Random RT-PCR amplification using the WTA kit was at least one log higher than that using the conventional random hexamer (data not shown). This suggests that further improvement is required for whole viral genome analysis, although our system is suitable for the comprehensive detection of viral genes. In addition, the TG (CA)–rich bias was observed within the viral genome; therefore, it seems unlikely that the bias leads to quantitative differences of the detected sequences with respect to the original population.

Table 5. Summary of bacterial and human-derived gene analysis.

Sample	#F1	#F2	#F3		
Total reads	30,958	25,119	21,858		
Human	26,957 (100%)	23,029 (100%)	19,612 (100%)		
rRNA	252 (0.93%)	31 (0.13%)	203 (1.04%)		
coding region[a]	637 (2.36%)	298 (1.29%)	449 (2.29%)		
non-coding region[b]	21,208 (78.7%)	18,035 (78.3%)	15,226 (77.6%)		
Sample	**#N1**	**#N2**	**#N3**	**#N4**	**#N5**
Total reads	15,298	32,335	25,500	18,014	28,823
Bacteria	10,963 (100%)	14,423 (100%)	3,039 (100%)	9,180 (100%)	23,955 (100%)
rRNA	5,994 (54.7%)	10,076 (69.9%)	1,829 (60.2%)	5,424 (59.1%)	13,772 (57.5%)

[a]Hit reads with exon region.
[b]Hit reads with intron and intergenic regions.
doi:10.1371/journal.pone.0004219.t005

Almost all diagnostic NATs require viral genome information, and thus cannot be performed for novel or unexpected viral infections. In this study, we showed that a diagnostic system based on parallel high-throughput sequencing is useful for the direct detection of unknown and/or small numbers of viruses, as well as for the genetic characterization of major pathogenic viruses in clinical specimens. We plan to share this system domestically as well as with the Asian epidemic network (The Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases; http://www.crnid.riken.jp), in order to enable the earlier identification of unknown pathogens in a novel outbreak or bioterrorism.

The cost of this approach will be a key concern for its adaption by the research community. Microbe-derived DNA/RNA enrichment [33], with suitable elimination of host-derived genes as described above, could reduce the required number of reads per sample. In addition, parallel tagged sequencing [6] using sample-specific barcoding adaptors with 5′-nucleotide tagged PCR primers [34] would enable the analysis of multiple samples in a single sequencing region. If these methods were combined, it would lead to significant reductions the operating costs (i.e., $2,000 per sample) of 90% or more.

This system, which can produce >0.4 million clones per run within a half-day, could also be very useful for the rapid identification of important mutation(s) by direct comparison with wild and mutant viruses, including "pandemic Flu" [35] and more virulent noroviruses [36,37].

Materials and Methods

RNA isolation from clinical samples

We analyzed unlinked, anonymous samples in the Osaka Prefectural Institute of Public Health. The samples were nasopharyngeal aspirates and stools (n = 3 and 5, respectively) isolated during 2005–2007 in Osaka, Japan. Seasonal influenza A virus (Flu) in nasopharyngeal aspirates from 3- to 7-year-old children was detected by a rapid diagnostic kit (immunochromatography) using Flu-specific antibodies. In 2006/2007, a large-scale norovirus outbreak occurred in Osaka, Japan, mainly infecting patients in nursing homes and welfare facilities (53%), hospitals (27%), kindergartens (15%), and elementary and junior high schools (5%) [26]. #N1 to #N3 samples were collected during this outbreak. #N1 sample was derived from a hospitalized patient and #N2 and #N3 samples were derived from patients in a welfare facility in 2006 (October to December). #N4 sample was a kindergarten student when an outbreak occurred at the elementary school of its elder brother and sister. In contrast to these four cases of putative human-to-human transmission, #N5 sample was oyster-associated. #N4 and #N5 samples were collected in May 2005 and January 2006, respectively. Diagnosis of norovirus infection was based on RT–PCR [26]. The collected stool was suspended with an equal amount of PBS and was centrifuged at 15,000 rpm for 10 min. The supernatants (0.25 ml) were used for RNA isolation. This study was approved by the ethical review committees of the RIMD, Osaka University, Osaka Prefectural Institute of Public Health, National Institute of Infectious Diseases, and RIKEN.

Quantitative RT-PCR of norovirus

RNA extraction was performed using a Magtration-MagaZorbRNA Common kit (Precision System Science) and the viral copy number of norovirus was estimated with One-step real time RT-PCR [26] using a One-Step Realtime PCR reagent kit (Toyobo). A plasmid containing the target sequence was used as a control.

Random RT-PCR amplification

Total RNA was extracted from specimens with TRI-LS (Sigma-Aldrich), and was reverse-transcribed with the Transplex whole transcriptome amplification (WTA) kit (Sigma-Aldrich) [21] using a quasi-random primer, according to the manufacturer's protocol. PCR amplification for the preparation of template DNA for pyrosequencing was carried out by AmpliTaq Gold DNA Polymerase LD (Applied Biosystems) [21]. Norovirus-specific PCR was performed as described above [26], and Flu-specific PCR was performed using the FluA M gene-specific primer set (M30F: 5′-TTCTAACCGAGGTCGAAACG-3′ and M264R2: 5′-ACAAAGCGTCTACGCTGCAG-3′).

RT- PCR diagnosis

Viral RNA was extracted from nasopharyngeal and fecal specimens with a QIAamp Viral RNA Mini Kit (QIAGEN), and cDNA was synthesized using SuperScript™ III reverse transcriptase (Invitrogen) with a random hexamer, as described previously [21]. The generated cDNA was subjected to PCR using the Expand High Fidelity^PLUS PCR System (Roche) with primer sets specific to viruses, such as human coronaviruses [38], WU polyomavirus [24], and PMMV [31].

Pyrosequencing and data analysis

The amplified cDNA was used as a template for GS FLX analysis (454 Life Sciences). A 70×75 PicoTiterPlate device (gasket for 16 regions) was divided into 2 regions for each of 8 samples. The obtained data were then subjected to a data analysis pipeline. Data analysis was performed on each read sequence by computational tools, as constructed previously [20] with some modifications. The analysis steps were: (i) remove tag sequences; (ii) execute a BLASTN search by Hi-per BLAST (Fujitsu); (iii) identify the scientific name for each read based on the NCBI taxonomy database; (iv) extract viral reads and perform mapping to reference data by SSEARCH. This analysis pipeline was constructed by utilizing BioRuby [39], BioPerl [40], and MySQL. After classification, particular human and bacterial reads were further analyzed as follows. Human genome mapping was performed by MEGABLAST search against the Human Genome, Homo_sapiens.NCBI36.49, using a threshold of 1E-40. Bacterial rRNA typing was performed by BLASTN search against the comprehensive rRNA database "silva" release 94 [41] using a threshold of 80% match per read.

Supporting Information

Figure S1 Cover depth of norovirus. Norovirus Hu/NLV/Oxford/B2S16/2002/UK (NCBI accession number: AY587989) was used as a reference sequence.
Found at: doi:10.1371/journal.pone.0004219.s001 (0.21 MB PDF)

Table S1 Summary of the best hits for each query sequences (E-value<1E-40) in nasopharyngeal aspirates
Found at: doi:10.1371/journal.pone.0004219.s002 (0.15 MB PDF)

Table S2 Summary of the best hits for each query sequences (E-value<1E-40) in fecal samples
Found at: doi:10.1371/journal.pone.0004219.s003 (0.20 MB PDF)

Table S3 Bias towards TG(CA)-enrichment within the detected norovirus sequences in fecal samples
Found at: doi:10.1371/journal.pone.0004219.s004 (0.10 MB PDF)

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Author Contributions

Conceived and designed the experiments: SN NS YO JK YH YN TH TI TN. Performed the experiments: SN CSY MU TT MT NM JK TI TN. Analyzed the data: SN AY NG TY MT RM. Contributed reagents/materials/analysis tools: NS KT TM TI. Wrote the paper: SN CSY NS TT KI TN.

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

Shumpei Watanabe[a], Tetsuya Mizutani[b,*], Kouji Sakai[b], Kentaro Kato[a], Yukinobu Tohya[a], Shuetsu Fukushi[b], Masayuki Saijo[b], Yasuhiro Yoshikawa[c], Ichiro Kurane[b], Shigeru Morikawa[b], Hiroomi Akashi[a]

[a] *Department of Veterinary Microbiology, Graduate School of Agricultural and Life Sciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan*

[b] *Department of Virology 1, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashimurayama, Tokyo 208-0011, Japan*

[c] *Department of Biomedical Science, Graduate School of Agricultural and Life Sciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan*

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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.

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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

* Corresponding author. Tel.: +81 42 561 0771; fax: +81 42 565 3315.
E-mail address: tmizutan@nih.go.jp (T. Mizutani).

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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 × *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.

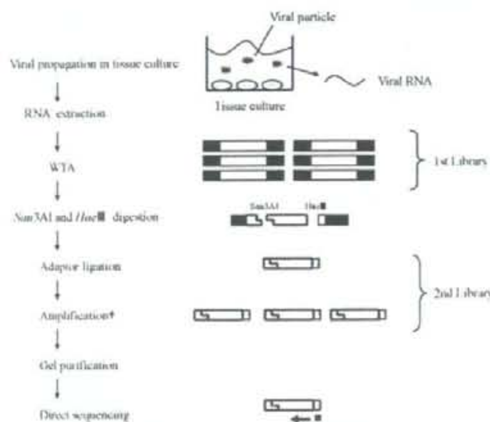

Fig. 1. Overall scheme for RDV ver3.0. WTA, whole transcriptome amplification; (†) with specially designed primer sets as shown in Fig. 2B.

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.

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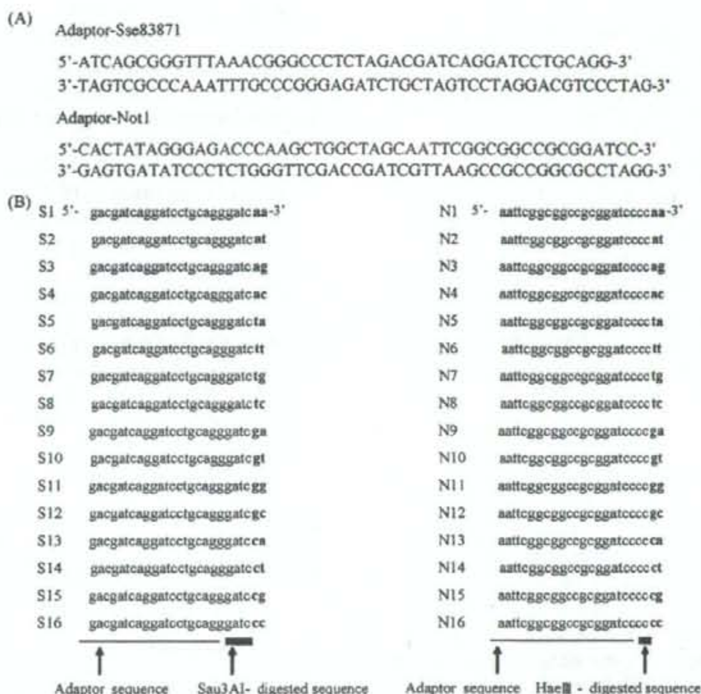

Fig. 2. The design of adaptors and primer sets used in RDV ver3.0. (A) Adaptor-Sse83871 contains sticky-end structures digested with Sau3AI, whereas Adaptor-NotI contains blunt-end structures digested with HaeIII. (B) All primers used in RDV ver3.0. Bold letters indicate 2-variable nucleotides.

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

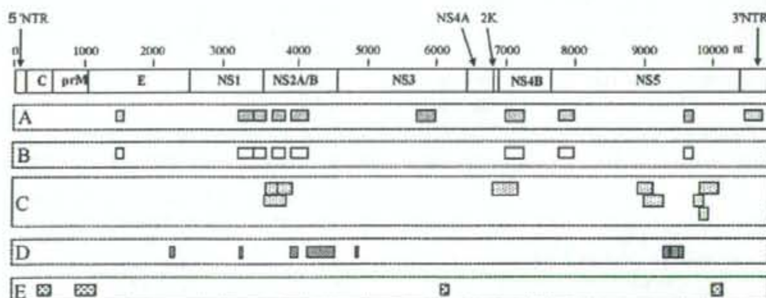

Fig. 3. Location of viral cDNA fragments on YOKV genome amplified using the RDV ver3.0. Column A shows 10 cDNA fragments expected to be detected in RDV ver3.0 (over 150 bp cDNA fegments digested by Sau3AI and HaeIII). Columns B–E show YOKV cDNA fragments detected in RDV ver3.0. Column B shows 8 of 10 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.

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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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BRIEF REPORT

Novel virus discovery in field-collected mosquito larvae using an improved system for rapid determination of viral RNA sequences (RDV ver4.0)

Takuya Yamao · Yuki Eshita · Yuki Kihara · Tomomitsu Satho · Makoto Kuroda · Tsuyoshi Sekizuka · Miho Nishimura · Kouji Sakai · Shumpei Watanabe · Hiroomi Akashi · Yupha Rongsriyam · Narumon Komalamisra · Raweewan Srisawat · Takeshi Miyata · Akira Sakata · Masato Hosokawa · Manabu Nakashima · Nobuhiro Kashige · Fumio Miake · Shuetsu Fukushi · Mina Nakauchi · Masayuki Saijo · Ichiro Kurane · Shigeru Morikawa · Tetsuya Mizutani

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Abstract In this study, we improved a method for rapid determination of viral RNA sequences (RDV) to overcome the limitations of previous versions. The RDV ver4.0 method can detect RNA sequences with at least 1,000 copies as starting material. A novel virus, which was isolated from field-collected *Aedes aegypti* larvae in the Phasi Charoen district of Thailand using C6/36 cells, was identified using the RDV ver4.0 protocol. The virus was named Phasi Charoen virus (PhaV). We used a high-throughput pyrosequencing approach to obtain more information about the genome sequence of PhaV. Analysis of a phylogenic tree based on amino acid sequences strongly suggested that PhaV belongs to the family *Bunyaviridae*.

T. Yamao · Y. Kihara · T. Satho · M. Nishimura · T. Miyata · A. Sakata · M. Hosokawa · M. Nakashima · N. Kashige · F. Miake
Faculty of Pharmaceutical Sciences, Fukuoka University, 8-19-1, Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan

Y. Eshita
Department of Infectious Diseases, Faculty of Medicine, Oita University, 1-1 Idaigaoka, Hasama, Yufu-shi, Oita 879-5593, Japan

M. Kuroda · T. Sekizuka
Laboratory of Bacterial Genomics, Center for Pathogen Genomics, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

K. Sakai · S. Fukushi · M. Nakauchi · M. Saijo · I. Kurane · S. Morikawa · T. Mizutani (✉)
Virology 1, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashimurayama, Tokyo 208-0011, Japan
e-mail: tmizutan@nih.go.jp

S. Watanabe · H. Akashi
Department of Veterinary Microbiology, Graduate School of Agricultural and Life Sciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

Y. Rongsriyam · N. Komalamisra · R. Srisawat
Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University, 420/6 Rajvithi Road, Bangkok 10400, Thailand

Mosquitoes transmit various pathogenic microorganisms, including viruses and parasites. The epidemic areas of mosquito-borne disease are predicted to spread due to the difficulty of controlling mosquito populations and widening mosquito distribution as a result of global warming. For example, the geographic distribution of *Aedes aegypti*, which transmits several flaviviruses such as yellow fever and dengue virus, has spread northward in North America [14]. The surveillance of mosquito-borne disease is dependent upon determining the viral infection transmitted by mosquitoes. Research has focused on the development of viral detection tools using reverse transcription-polymerase chain reaction (RT-PCR). Recently, we developed a rapid determination system for viral RNA sequences (called RDV) that is useful for determining a viral genome sequence without cloning in a plasmid vector [11]. In addition, the RDV method allows exhaustive identification of viruses in comparison with previous viral detection systems such as RT-PCR because a primer specific for a target viral nucleotide sequence is not used in RDV. In our previous research, RDV version 1 (RDV ver1.0) was used to detect some mosquito-borne RNA viruses, such as West Nile virus, Japanese encephalitis virus, and dengue virus type 2, from cell culture supernatant [11]. We applied RDV

ver1.0 to homogenates of *Aedes aegypti* adult females collected from a dengue epidemic area in Thailand, using the mosquito cell line C6/36. Co-infection of dengue virus type 4 and cell fusing agent virus was detected [7]. To increase sensitivity over RDV ver1.0, the sequence-independent amplification step was improved (RDV ver2.0), and avian paramyxovirus was detected in the allantoic fluid of embryonated chicken eggs [13]. In the RDV ver2.0 method, a multiplex PCR system (Takara Bio Inc., Japan) was used, and many amplicons were obtained at the final step. The AmpliTaq Gold PCR system (Applied Biosystems, USA) was used in RDV ver2.1 instead of the multiplex PCR system. A new adenovirus, Ryukyu virus (RV) 1, belonging to the family *Adenoviridae*, which was isolated from *Pteropaus dasymallus yayeyamae*, was successfully detected in the culture supernatant of primary kidney cells using the RDV method (RDV-D) [10]. We further developed the RDV method to produce RDV ver3.0, with the number of primer sets reduced to 256 [16]. The sensitivity of these RDV methods was approximately 10,000 copies per reaction. In addition, short-length RNA (<1 kb) was difficult to amplify. Therefore, in this study, we further improved the RDV method for detecting a wide range of viral genomic RNA and to increase the sensitivity of amplification.

Short-length RNA (<1 kb) is difficult to amplify by using the whole transcriptome amplification kit (Sigma-Aldrich), REPLI-g kit (Qiagen), and Genomiphi V2 kit (GE Healthcare) due to the use of quasi-random or random primers and/or nuclease activity, whereas the QuantiTect whole transcriptome kit (Qiagen) improves the problem by using ligation of cDNA. This kit is optimized for whole transcriptome amplification and delivers high cDNA yields by following a simple three-step protocol. RNA is first transcribed to cDNA using T-Script reverse transcriptase. The cDNA is ligated using a high-efficiency ligation mix and then amplified using REPLI-g. In this study, SuperScript III (Invitrogen) as a reverse transcriptase and GenomiPhi V2 for sequence-independent amplification of cDNA were applied to the QuantiTect whole transcriptome kit. The results showed that although a simple three-step process was not achieved due to a different buffer system, the sensitivity was increased compared with the original protocol by Qiagen as described below. To further increase sensitivity, a 5′phosphorylated 20 mer oligonucleotide was added at the ligation step. The improved RDV method developed in this study, with increased sensitivity, is called RDV ver4.0 (Fig. 1).

The RDV ver4.0 method includes the following four procedures (Fig. 1).

(1) RNA extraction was described in the original RDV ver1.0 method [11]. (2) cDNA synthesis was also described in the RDV ver1.0 method. cDNA was synthesized using random hexamer [11]. In order to amplify a low amount of short-length cDNA using RDV ver4.0, oligonucleotide was added at the ligation step for constructing bridges amongst cDNAs. Ligation buffer and enzyme from the QuantiTect Whole Transcriptome kit (Qiagen) was added to 10 μl of cDNA in the presence of 5 μl of 100 μM oligo-dA (5′-P-AAAAAAAAAAAAAAAAAAA-3′) or oligo-1 (5′-P-GTNNNANNCGNNNGTNNNAN-3′). After the reaction mixture was incubated at 22°C for 2 h, 1 μl of ligation solution was amplified using Phi29 DNA polymerase (GenomiPhi V2 DNA amplification kit) at 30°C for 2 h (first cDNA library). (3) Construction of the second cDNA library was described in RDV ver1.0 method [11]. In this study, DNA was digested with *Hae*III or *Alu*I (Takara Bio Inc.). Ligation-convenience kit (Nippon Gene, Tokyo, Japan) was used for adaptor ligation. The second cDNA library was amplified by PCR using specially designed primer sets [11]. (4) Direct sequencing [11].

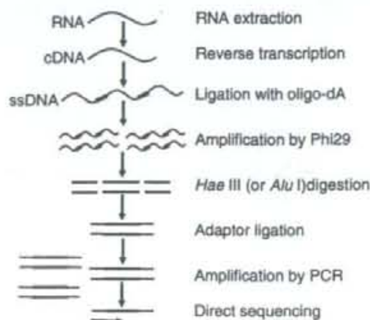

Fig. 1 Overall scheme for RDV ver4.0

To investigate the sensitivity of RDV ver4.0 to short-length RNA, in vitro-synthesized albumin mRNA was used as the template. RDV ver4.0 has the potential to detect at least 1,000 copies of short-length RNA (data not shown). The RDV ver4.0 method was successfully used to detect dengue fever virus type 4, cell fusing agent virus and Yokose virus (data not shown).

Aedes aegypti larvae were collected at the homes of dengue fever patients at Phasi Charoen, Bang Khun Thian, Bang Khae, Bang Bon and Chom Thong Districts in Bangkok Province, Thailand, in May 2007. The larvae were homogenized in 200 μl of MEM with 2% FBS. Each homogenate was centrifuged at 550 *g* for 10 min at 4°C, and supernatant was filtered through 0.22 μm Millex-GX filters (Millipore, Billerica, MA, USA). The supernatant (25 μl) of 33 groups of field-collected mosquito larvae was exposed to the C6/36 cells in a 24-well plate. After 8 days, a cytopathic effect (CPE) was observed in 14 groups. In particular, group number 12 exhibited a strong CPE. The supernatant of group number 12 was collected and was

used for RDV ver4.0 after centrifugation at 550 *g* for 10 min at 4°C. A total of 149 PCR products at the final step of RDV ver4.0 were extracted from agarose gels, and direct sequencing was performed using forward primers. Each nucleotide sequence was used to determine homologous sequences using blastx on the National Center for Biotechnology Information (NCBI) website. Four read sequences, which consisted of 37 amino acids, were found to have low homology with a sequence of RNA-dependent RNA polymerase of rice stripe virus (RSV; GenBank accession number EF141327) (Fig. 2a, b). The amino acid sequence also had low homology to Uukuniemi virus (UUKV) (accession number D10759) and Rift Valley fever virus (RVFV) (accession number Z30318) of the genus *Phlebovirus* (Fig. 2b).

To eliminate the possibility that the RSV-like sequence originated from C6/36 cellular DNA and RNA, mock-infected cells were used as negative controls. RNA and DNA were prepared from mock-infected C6/36 cells at Oita University and Nagasaki University. Primers (P1-3: 5′-GAACATATGAAGTTCTGCAA-3′ and P2-2: 5′-GCAACCTAATAATCCAGGGC-3′) were designed for amplification of the RSV-like sequence. The expected size of PCR product was 92 bp. No amplification was observed for these C6/36 cells (data not shown). To investigate how many mosquito larva groups have RSV-like RNA in homogenate-inoculated cells, PCR was performed for amplification of RSV-like sequences. Eighteen of 33 groups comprised the RSV-like sequence (data not shown). In addition, there was no relationship between CPE and the appearance of an RSV-like sequence.

To investigate the infectivity of the RSV-like agent, the supernatant of C6/36 cells inoculated with homogenate from group number 12 was inoculated onto fresh Vero (African green monkey kidney) cells and C6/36 cells (second passage). After 5 days, RNA was extracted from the supernatant and PCR was performed. However, an RSV-like fragment was not detected in either of the cell lines (data not shown). After 10 days, an RSV-like fragment was amplified in the supernatant RNA of C6/36 cells, but not of Vero cells. The supernatant of C6/36 cells inoculated with PCR-positive group number 12 was inoculated onto fresh C6/36 cells (third passage), and RNA was extracted from cells and supernatant after 10 days. RNAs extracted from C6/36 cells and supernatant were positive for the RSV-like fragment. The supernatant of C6/36 cells inoculated with group number 12 homogenate was further inoculated onto fresh C6/36 cells (fourth passage), and RNA was extracted after 10 days. The RSV-like fragment was detected in both cells and supernatant (data not shown). These results indicated that the RSV-like agent was infectious to C6/36 cells. We named this RNA virus Phasi Charoen virus (PhaV). PhaV does not induce CPE after the second passage. Therefore, there may be another virus that causes CPE in C6/36 cells in group number 12. This virus may replicate slowly compared to PhaV, or PhaV may have mutation(s) in genes that are responsible CPE after the first passage.

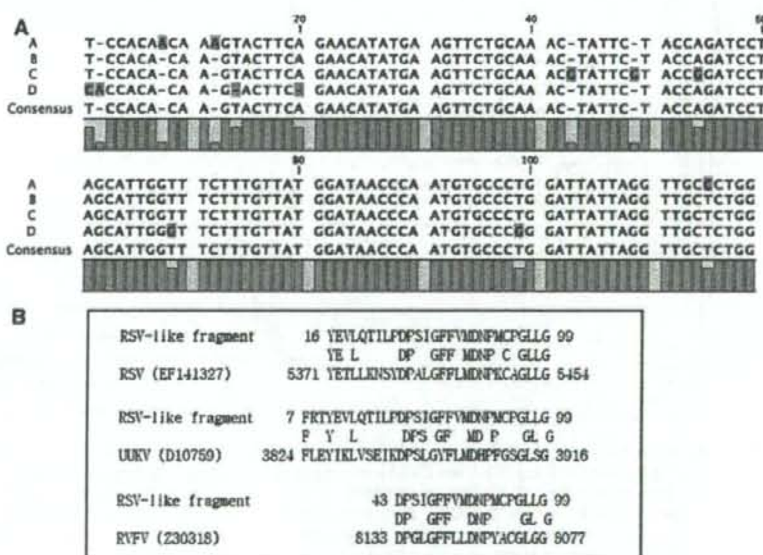

Fig. 2 Rice stripe virus-like sequence obtained using RDV ver4.0. **a** Alignment of four nucleic acid sequences (**a–d**) obtained from number 12 homogenate using RDV ver4.0. **b** Homology of RSV-like sequence to RSV, Uukuniemi virus (UUKV), and Rift Valley fever virus (RVFV)

To obtain information regarding the genome sequence of PhaV, we used the Genome Sequencer FLX System of Roche and 454 Life Sciences. The cDNA synthesized from RNA in the second passage supernatant, as described above, was amplified by using GenomiPhi V2. DNA sequencing libraries for Genome Sequencer FLX were constructed and sequenced by Takara Bio Inc. The obtained 5,000 read sequences were analyzed by Phred/

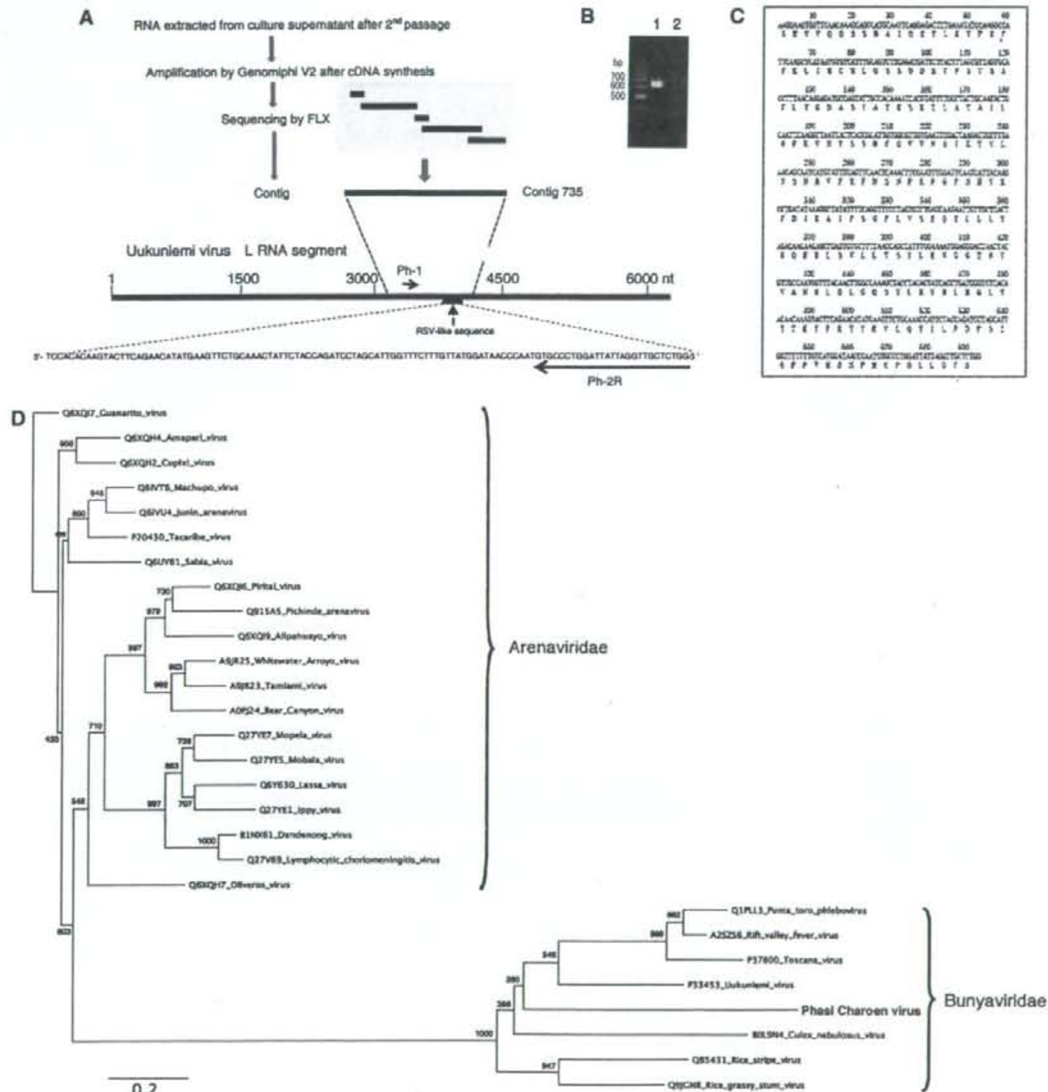

Fig. 3 Phylogenetic tree analysis of PhaV. a Contig 735 was located on the UUKV L segment. Primers (Ph-1 and Ph-2R) were designed for amplification within contig 735. b cDNA from supernatant of the fourth passage was amplified (lane 1). cDNA from mock-infected supernatant was used as a control (lane 2). c Nucleic acid sequence of the PCR product and amino acid sequence encoded by the nucleic acid sequence. d The phylogenetic tree was obtained by using the neighbor-joining method with 1,000 bootstrap replicates, and branch length is shown at each branch node. The horizontal scale indicates 0.2 amino acid substitutions per site. Arenaviruses were added to this phylogenetic tree as an out-group of segmented, negative-stranded RNA virus

Phrap/Consed for de novo assembly [6]. Homology searches were carried out using the blastn and blastx programs against non-redundant nucleotide and protein databases, respectively [1]. Read sequences were assembled, and several contigs that indicated homology to viral sequences were obtained (Fig. 3a). One of the contigs contained the RSV-like sequence, and two primers were designed (Ph-1 primer: 5′-CAGGGGATCTTACACTATACATCATCCC-3′ and Ph-2R primer: 5′-CCAGGGCAACCTAATAATCCA GGGCAC-3′). The Ph-2R primer contained the RSV-like sequence. The cDNA synthesized from supernatant RNA of C6/36 cell culture (fourth passage) was PCR-amplified using Phusion Flash High-Fidelity PCR master mix (Finnzymes, Espoo, Finland), and a band was detected on an agarose gel (Fig. 3b). After electrophoresis, the band was purified from the gel, and sequencing was performed using both Ph-1 and Ph-2R primers. Sequence data (Fig. 3c) was deposited in DDBJ/EMBL/GenBank (accession number AB441720). To retrieve homologs with the PhaV sequence, the partial amino acid sequence of PhaV was analyzed for homolog clustering using FlowerPower V2 with the global-local homology parameter [8]. The phylogenetic tree of the amino acid sequences was obtained by using the neighbor-joining method in the ClustalX program with 1,000 bootstrap replicates [15]. The phylogenetic tree based on amino acid sequences suggested that PhaV belongs to the family *Bunyaviridae* (Fig. 3d).

The family *Bunyaviridae* consists of more than 350 viruses and is divided into five genera: *Hantavirus*, *Nairovirus*, *Orthobunyavirus*, *Phlebovirus*, and *Tospovirus* [3]. PhaV is thought to be closely related to culex nebulosus virus, which is an unclassified member of the family *Bunyaviridae*. Amongst the members of the genus *Phlebovirus* of the family *Bunyaviridae*, UUKV is comparatively closely related to PhaV 1, but RVFV shows low homology to PhaV. Interestingly, a 12-aminoacid sequence motif, DPXLGXFLXDXP, is conserved between RSV, UUKV and RVFV. This motif may be important for the function of RNA-dependent RNA polymerase in these viruses. The majority of members of the family *Bunyaviridae* are transmitted by arthropods [4]. UUKV is transmitted by *Ixodes ricinus* ticks [12], whereas RVFV is transmitted by mosquitoes [5]. Tahyna virus infects vertically in *Aedes aegypti* [9]. RVFV are important human pathogens, but there is no report regarding the association of UUKV and human disease except for production of antibodies against UUKV in humans [2]. In this study, we show that PhaV was isolated from the mosquito cell line C6/36, but not from the mammalian Vero cell line. Therefore, PhaV may have non-pathogenic properties in mammalian cells, similar to UUKV. However, there is a possibility that Vero cells are susceptible to PhaV during a longer inoculation period.

In this study, we were able to increase the sensitivity of the RDV method, and we used the new RDV ver4.0 for the detection of a nucleotide sequence of a novel mosquito virus, PhaV. The RDV ver4.0 method will be useful for greatly improved sequence-independent detection of RNA viruses, especially when emerging virus disease occurs.

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M. genavense infections occur only rarely in persons other than AIDS patients (as in the present case), but they always occur in immunocompromised persons (*7,8*). To date, only 1 case of disseminated infection has been reported in a solid-organ (kidney) transplant recipient; the diagnosis was made by molecular identification in isolates from blood and marrow cultures. That patient died of complications from *M. genavense* infection (*9*). Because *M. genavense* is a fastidious organism, the infections it causes are difficult to diagnose and their frequency is probably underestimated, which may change with increased use of direct molecular biological methods.

Optimal treatment of *M. genavense* infections has not been established (*10*). Experience with *M. genavense* infections in AIDS patients and with other nontuberculous mycobacteria infections in solid-organ transplant recipients suggests that at least 2 antimicrobial drugs should be used for a prolonged period; when possible, immunosuppressive drugs should be concurrently reduced (*1,3,6,10*). Outcome of nontuberculous mycobacteria infections in transplant patients is highly variable (*1,5*) but was satisfactory in the present patient, who was treated with quintuple antimicrobial-drug therapy and reduced immunosuppressive therapy.

This case of a disseminated infection due to *M. genavense* in a heart transplant recipient was diagnosed early. Universal 16S rRNA gene sequencing after amplification directly from intestinal biopsy specimens enabled fast diagnosis and appropriate management.

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Victoire de Lastours,*† Romain Guillemain,*† Jean-Luc Mainardi,*†‡§ Agnès Aubert,*† Patrick Chevalier,*† Agnès Lefort,*¶ and Isabelle Podglajen,*†‡§

*Assistance Publique-Hôpitaux de Paris, Paris, France; †Hôpital Européen Georges Pompidou, Paris, France; ‡Université Paris-Descartes, Paris, France; §Université Pierre et Marie Curie, Paris, France; and ¶Hôpital Beaujon, Paris, France

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Address for correspondence: Isabelle Podglajen, Assistance Publique–Hôpitaux de Paris, Hôpital Européen Georges Pompidou, Service de Microbiologie, 20 rue Leblanc, 75908 Paris CEDEX 15, France; email: isabelle.podglajen@hop.egp.ap-hop-paris.fr

Isolation of Novel Adenovirus from Fruit Bat (*Pteropus dasymallus yayeyamae*)

To the Editor: Bats are thought to be one of the most important reservoirs for viruses such as Nipah virus, severe acute respiratory syndrome (SARS) coronavirus, and Ebola virus (*1*). These pathogens became known after extensive surveys of bats following outbreaks. As a first step in investigating unidentified pathogens in bats and to help forecast the potential threat of emerging infectious diseases, we tried to isolate and characterize viruses that persistently infect bats. In the process, we isolated a novel adenovirus from a fruit bat in Japan.

Pteropus dasymallus yayeyamae, or Ryukyu flying fox, is a fruit bat of Japan. With the permission of the governor of Okinawa, we caught 1 adult male bat of this species and used its spleen and kidneys to establish primary cell cultures. On the 4th passage of the primary adherent cells derived from the spleen, a cytopathic effect (CPE) appeared without any visible

microbe, indicating that the cell culture contained a virus. The virus, tentatively named Ryukyu virus 1 (RV1), caused apparent CPE on primary kidney cells derived from a Ryukyu flying fox and on our established bat kidney T1 (BKT1) cells, which were derived from the kidney of a horseshoe bat (*Rhinolophus ferrumequinum*) and transformed with expression plasmid DNA encoding the large T antigen of replication origin-defective simian virus 40.

To identify the virus, RV1, we applied the rapid determination of viral RNA (RDV) system version 1.0 (*2*). However, no viral nucleic acid sequence was detected from an RNA sample in the RV1-infected BKT1 cells. For detection of viral DNA, we developed a system for rapid determination of viral DNA sequences (RDV-D) by minor modification to the RDV system for RNA viruses (*2–4*). The results indicated that 2 of the fragments were homologous to the gene encoding the precursor of terminal protein (pTP) of adenoviruses. Further RDV-D analysis showed that 6 fragments (139 bp, DDBJ/EMBL/GenBank accession no. AB302970) were homologous to the pTP gene and that another 6 fragments (316bp, DDBJ/EMBL/GenBank accession no. AB302971) were homologous to the gene encoding the precursor of protein VI (pVI) of adenoviruses. These results indicated that RV1 must belong to the family *Adenoviridae*.

To further confirm that RV1 isolate was an adenovirus, we used PCR and sequencing. We performed the first reaction with the outer primer pair (polFouter and polRouter) of a nested PCR method, targeting the viral DNA polymerase gene with highly degenerate consensus primers that have been described recently (*5*). A fragment of ≈550 bp was amplified from RV1 as well as from human adenoviruses-1, -3, -4, and -7 (data not shown). Sequence analysis of the amplified product (DDBJ/EMBL/GenBank accession no. AB303301) showed that RV1 was homologous to tree shrew adenovirus 1 (70.0% amino acid sequence identity), porcine adenovirus 5 (69.2%), canine adenovirus 1 (68.9%), human adenoviruses-3, -16, -21 and -50 (68.9%), and other viruses (>64.8%) in genus *Mastadenovirus*, but less homologous (46.7%–57.8%) to viruses in other genuses, *Siadenovirus*, *Aviadenovirus*, and *Atadenovirus*. In addition, a phylogenic tree based on amino acid sequences indicated that RV1 belongs to family *Adenoviridae*, genus *Mastadenovirus* (Figure).

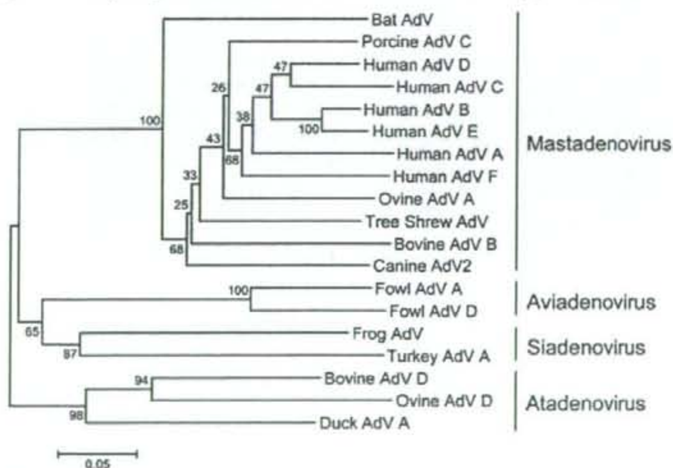

Figure. Phylogeny of adenoviruses based on analysis of partial amino acid sequences of DNA polymerase protein. Trees were estimated by using the neighbor-joining method based on the amino acid pairwise distance and MEGA 4.0 software (www.megasoftware.net). Numbers represent percentage bootstrap support (100 replicates).

Electron microscopy of RV1-infected BKT1 cells indicated that RV1 accumulated in the nucleus and that the size of capsids was 60–70 nm (data not shown). Restriction endonuclease analysis of the RV1 genome indicated that the genome was ≈20–30 kbp (data not shown). These features are consistent with RV1 being an adenovirus.

Until now, a number of RNA viruses have been isolated from bats, but isolation of DNA virus is rare (*1*). The isolation of the novel adenovirus seems to be possible because of usage of the primary cells originated from the host; DNA viruses might have more restricted host range than RNA viruses and require host-originated cells for the growth. In addition, our success in DNA virus isolation might have resulted from usage of the adult animal latently and persistently infected with DNA viruses such as adenovirus and herpesvirus.

In conclusion, we isolated a novel virus from a fruit bat. This virus was isolated from a healthy bat, which suggests that the virus may persistently infect fruit bats. Although its pathogenicity for humans is still unknown, knowledge of RV1 will be useful in epidemiologic studies of infectious diseases emerging from bats because persistently infecting viruses might be isolated together with primary pathogens. We are planning to establish cell lines from bats and isolate more viruses from persistently infected bats.

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Ken Maeda,* Eiichi Hondo,* Junpei Terakawa,* Yasuo Kiso,* Numekazu Nakaichi,* Daiji Endoh,† Kouji Sakai,‡ Shigeru Morikawa,‡ and Tetsuya Mizutani‡

*Yamaguchi University, Yamaguchi, Japan; †Rakuno Gakuen University, Ebetsu, Japan; and ‡National Institute of Infectious Diseases, Tokyo, Japan

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Address for correspondence: Ken Maeda, Laboratory of Veterinary Microbiology, Faculty of Agriculture, Yamaguchi University, 1677-1 Yoshida, Yamaguchi 753-8515, Japan; email: kmaeda@yamaguchi-u.ac.jp

Letters

Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have one Figure or Table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Fluoroquinolone-Resistant Group B Streptococci in Acute Exacerbation of Chronic Bronchitis

To the Editor: Fluoroquinolones (FQs) that are active against streptococcal species (e.g., levofloxacin and moxifloxacin) have been recommended by numerous national health authorities and international organizations for treating acute exacerbations of chronic bronchitis and pneumonia in adults (*1*). However, use of these antimicrobial drugs for treating community-acquired infections has led to an increase in FQ-resistant strains in bacteria such as *Streptococcus pneumoniae*. Group B streptococci (GBS, e.g., *S. agalactiae*) are the leading cause of invasive infections (pneumonia, septicemia, and meningitis) in neonates. GBS are also associated with bacteremia, endocarditis, and arthritis, and are responsible for deaths and illness in nonpregnant women with underlying diseases and in elderly adults (*2*). We describe, to our knowledge, the first GBS clinical isolate in France resistant to FQ; the isolate was from a patient treated with levofloxacin.

GBS CNR0717 strain was isolated as the predominant bacterium in a culture ($>10^7$ CFU/mL) from 2 purulent sputum samples from an 80-year-old man (leukocytes >25, epithelial cells <10) obtained 8 days apart. This patient was treated for 2 weeks with levofloxacin, 750 mg/day, for acute exacerbation of chronic bronchitis. No other relevant respiratory bacterial pathogens were present in these samples. GBS CNR0717, a capsular serotype IV strain, was suspected to have reduced susceptibility to FQs because no inhibition zone was observed around disks containing norfloxacin and pefloxacin disks, and reduced diameters were observed around disks containing ciprofloxacin and levofloxacin. Antibiograms were performed according to recommendations of the Clinical and Laboratory Standards Institute (*3*) on Mueller Hinton agar (Bio-Rad, Marnes la Coquette, France) supplemented with 5% horse blood. This strain was susceptible to all other antimicrobial drugs usually active against GBS (penicillin, erythromycin, clindamycin, tetracycline, rifampicin, vancomycin) and showed low-level resistance against aminoglycosides. MICs for 6 FQs (Table) indicate that GBS CNR0717 was highly resistant to pefloxacin and norfloxacin, with MICs >64 mg/L, and showed increased MICs for ciprofloxacin, sparfloxacin, levofloxacin, and moxifloxacin. No reduction of FQ MICs was observed with reserpine (10 mg/L), which indicated that resistance to FQ was not caused by an active efflux pump system.

Three major mutations have been reported for FQ resistance in streptococci at codon positions 81 in *gyrA* and 79 or 83 in *parC* (*4*). DNA sequence analysis of these regions showed a mutation in *parC* (Ser 79 → Tyr) but not in the wild-type susceptible strain (NEM316). No mutation was detected in the *gyrA* gene. FQ resistance in streptococci is acquired through a stepwise process and has been extensively studied in *S. pneumoniae*. First-step mutants conferring low-level resistance generally result from mutations in either *gyrA* or *parC*. There is also

Table. MICs of fluoroquinolones for strains of group B streptococci (GBS), France

Strain	MIC (mg/L)*					
	Pef	Nor	Cip	Spa	Lev	Mox
GBS CNR07017	>64	>64	4	1	4	1
GBS NEM316	16	8	2	0.5	1	0.25

*Pef, pefloxacin; Nor, norfloxacin; Cip, ciprofloxacin; Spa, sparfloxacin; Lev, levofloxacin; Mox, moxifloxacin.

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LETTER TO THE EDITOR

Transfusions of red blood cells from an occult hepatitis B virus carrier without apparent signs of transfusion-transmitted hepatitis B infection

Dear Sir

To minimize the risk of transfusion-transmitted hepatitis B virus (HBV) infections, the Japanese Red Cross (JRC) Blood Centers have adopted a multistep screening system to identify donors at risk of HBV infection. First, donors are examined for the hepatitis B surface antigen (HBsAg) by performing reverse passive haemagglutination tests with a sensitivity of 3 ng mL^{-1}. HBsAg-negative donations are screened for antibodies against HBsAg and the hepatitis B core antigen (anti-HBs and anti-HBc, respectively) by particle haemagglutination and haemagglutination inhibition (HI) tests, respectively. Donations with a high anti-HBs titre ($\geq 2^4$ dilution equivalent to 200 mIU mL^{-1}) or a low or zero anti-HBc titre ($\leq 2^4$ dilution) are defined as 'seronegative'. The cut off value for anti-HBc tests is relatively high compared to that of enzyme-linked immunoassays (EIAs) because HBV DNA was not detected by an in-house polymerase chain reaction (PCR) in donors who tested negative for HBsAg and positive for anti-HBc at an HI titre less than 2^5 (Iizuka *et al.*, 1992). Since the introduction of nucleic acid amplification test (NAT) technology, all seronegative donations are pooled (initially, at a pool size of 500 and a current pool size of 20, i.e. 20-NAT) and subjected to NAT (Ampli-NAT, Roche, IN, USA). If the 20-NAT tests positive, the pooled donations are further subjected to individual NAT (ID-NAT) to identify the blood donation that contains the viral genome. The 95% confidence interval of the detection range for HBV in ID-NAT is 22–60 copies of HBV per millilitre (Meng *et al.*, 2001). Donors who did not fall within the algorithm would be either categorized in the window period of 20 NAT or assigned an occult HBV status with a low viral load (reviewed by Raimondo *et al.*, 2007).

Correspondence: Rika A. Furuta, Osaka Red Cross Blood Center, 2-4-43 Morinomiya, Joto-ku, Osaka 536-8505, Japan.
Tel.: +81 6 6962 7066; fax: +81 6 6962 7029;
e-mail: furuta@osaka.bc.jrc.or.jp

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In November 2006, the Osaka Red Cross Blood Center, Japan, identified a repeat donor, namely, a 69-year-old female, whose donation was found to be positive for HBV DNA when tested by the latest 20-NAT. According to the guidelines for the safety of transfusion in the JRC Blood Centers, the serological status of the donation was re-evaluated. The donated blood was found to be negative for HBsAg, anti-HBs and anti-HBc by routine testing methods and positive for only anti-HBc when tested using EIA (AxSYM; Abbott Laboratories, Abbott Park, IL, USA), indicating that the donor was an occult HBV carrier with a low anti-HBc titre. We retrieved frozen aliquots of previous donations by this donor and found that sera donated on and after 1 October 1999 tested positive for HBV DNA when tested by ID-NAT. The amount of HBV DNA in these donations was less than 100 copies per millilitre, except for two donations (Table 1). From the 13 donations made by this donor in the abovementioned period, 11 components were transfused into recipients (recipient number 1–11 in Table 1). We collected the HBV test records of some of the recipients from the medical institutions where each recipient had been hospitalized. Recipients 3, 6, 7 and 9 had succumbed to their primary disease, and no records were available for recipients 10 and 11. Of the remaining five cases, the HBV test was performed at both the pre- and post-transfusion stages in recipients 1, 4 and 5, but recipients 2 and 8 were tested only at the post-transfusion stage. Recipient 1 was a 70-year-old female who had tested negative for HBsAg and anti-HBc by EIA 2 days prior to transfusion. She was transfused with packed red blood cells (RBCs) and tested negative for HBsAg, anti-HBs and anti-HBc by EIA and negative for HBV DNA by PCR 7 months after the transfusion. These data suggest that the latest RBC component from this occult HBV donor did not cause transfusion-transmitted HBV infection. In recipients 2 and 8, the post-transfusion EIA test results for HBsAg were reported negative. Recipient 4 tested negative for HBsAg by EIA at 11 days before

Table 1. HBV status of the donor and recipients

Donor					Recipients						
								Pretransfusion		Post-transfusion	
Date of donation	Pooled NAT	ID-NAT	Copy number per mL	Component	Recipient number	Age (years)	Primary diseases*	HBsAg	Anti-HBc	HBsAg	Other markers
1 November 2006	+†	+	ND	—¶							
22 May 2006	−†	+	<100	RBCs	1	70	(1)	−	−	−	−, anti-HBs; −, anti-HBc; −, HBV DNA
15 April 2006	−†	+	140	RBCs	2	NA	(2)	NA	NA	−	
26 September 2005	−†	+	210	RBCs	3	NA	NA	−	NA	—**	
27 June 2005	−†	+	<100	—¶							
10 April 2005	−†	+	<100	RBCs	4	86	NA	−	NA	−	−, HBV DNA
15 February 2004	−‡	+	<100	RBCs	5	60	(3)	−	NA	−	
15 September 2003	−‡	+	<100	—¶							
21 March 2003	−‡	+	<100	RBCs	6	69	(4)	−	NA	—**	
1 March 2002	−‡	+	<100	RBCs	7	51	(5)	NA	NA	—**	
1 July 2002	−‡	+	<100	RBCs	8	41	(6)	NA	NA	−	
15 January 2001	−‡	+	<100	RBCs	9	57	(7)	NA	NA	—**	
1 October 1999	−§	+	<100	RBCs	10	NA	NA	NA	NA	NA	
15 April 1999	−§	−	ND	RBCs	11	NA	NA	NA	NA	NA	

NA, not applicable; ND, not determined.
*Primary Diseases: (1), perforation of sigmoid diverticulum; (2), transverse colon cancer; (3), bleeding gastric ulcer; (4), operative diseases; (5) operative diseases; (6), gastric ulcer; and (7), ovarian cancer.
†20-pooled.
‡50-pooled.
§500-pooled.
¶Not used.
**Decease by the primary disease.

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transfusion with RBCs. Furthermore, she tested negative for HBsAg at both 17 and 19 months after the transfusion. In addition, PCR results for this patient were negative for HBV DNA 21 months after transfusion. In recipient 5, it was reported that both pre- and post-transfusion sera tested negative for HBsAg by EIA. Although no further reports suggesting any signs of HBV transmission in recipients 2, 4, 5, and 8 have been filed with our blood centre, the HBV test records of these four recipients are insufficient to determine whether transfusion-transmitted HBV infection occurred.

Kanagawa Red Cross Blood Center, Japan, recently reported a case of transfusion-transmitted HBV infection caused by an individual with an occult HBV infection who had repeatedly donated platelets and whose viral load fluctuated around the limit of HBV detection level by the ID-NAT (Inaba *et al.*, 2006). It is noteworthy that the component transfused in this case was a platelet concentrate containing approximately 200 mL of plasma; on the other hand, in our subjects, the transfused component was packed RBCs including 10–15 mL of plasma. A more recent look-back study on transfusion-transmitted HBV infection conducted by the JRC Blood Center identified that only one of the 33 components obtained from occult HBV donors caused the HBV infection (Satake *et al.*, 2007). This particular patient was transfused with 450 mL of fresh frozen plasma. The same study also demonstrated that 11 of the 22 components donated during the mini-pool NAT window period resulted in transfusion-transmitted HBV infection. Although the results of recipient 1 in our case appear to be consistent with those in the look-back study, data available in the literature suggest that occult HBV infection is transmissible, especially in endemic areas (reviewed by Liu *et al.*, 2006). To clarify the potential risks of blood components from occult HBV donors, many more cases need to be analysed in detail, where the total amount of HBV in the component transfused, the presence or absence of HBV antibodies in the component, the immunological status of the recipient, the HBV genotype and/or the presence of mutation(s) should be assessed.

The peculiar criterion of seronegative used in the JRC Blood Centers was a practical solution to exclude donors with a risk of HBV infection, without excessively reducing the size of the donor pool. This criterion was introduced because the prevalence of HBV infection, when serological testing was introduced, was relatively higher in Japan than in other industrialized countries. Our serological screening, however, has failed to identify a few occult HBV carriers with a low anti-HBc titre and a low viral DNA. JRC has been re-evaluating the efficacy of our screening strategy by follow-up surveys, including the present study, and exploring options to be adopted to minimize the risk not only by the occult HBV carrier but also by donors in the 20-NAT window period.

Although we consider that the current possibility of HBV transmission by occult HBV carriers with a low anti-HBc titre is limited in Japan, this consideration cannot be generalized to countries with different HBV prevalence as mentioned above. Once the cut off value of the anti-HBc titre confirming the HBV-DNA-negative status of the donor blood is more rigorously determined, our serological screening algorithm may be an acceptable option in areas of intermediate or high HBV endemicity where NAT is unavailable.

R. A. Furuta,* Y. Kondo,* T. Saito,*M. Tomita,*
K. Oka,* Y. Kishimoto,† Y. Tani* & T. Shibata*
**Osaka Red Cross Blood Center, Japanese Red Cross Society, Osaka, Japan and †Department of Hematology and Oncology, Kansai Medical University, Osaka, Japan*

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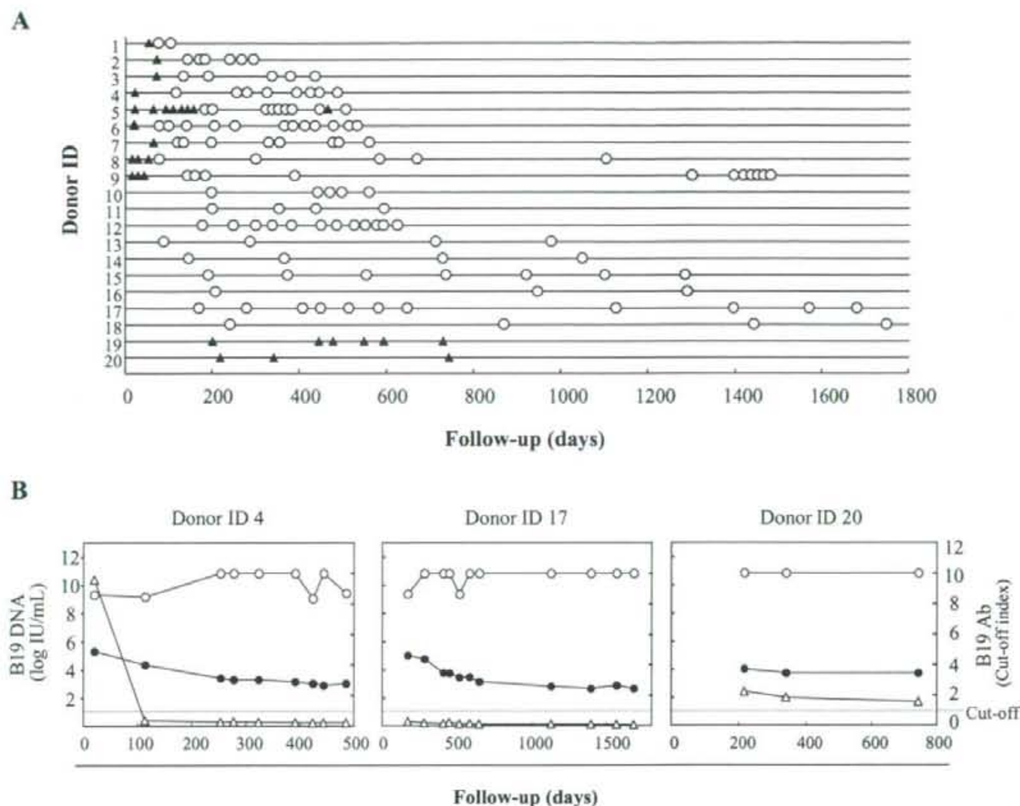

Fig. 2. (A) B19 IgM and IgG for individual donors at follow-up visits. (▲) Positive for both IgM and IgG; (○) positive for IgG. (B) Representative cases for three patterns of test results. Changes in viral load (●), IgM (△), and IgG (○). Donors correspond to those in A.

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New cell lines express HNA-1c, -4a, -4b, -5a, or -5b for identification of HNA antibodies

Antibodies to human leukocyte antigens (HLAs) or human neutrophil antigens (HNAs) are regarded to be the principal causes of nonhemolytic transfusion reactions, including transfusion-related acute lung injury. Although flow cytometric (FCM) analysis using panels of phenotyped neutrophils is widely used to detect and identify antibodies to HNAs, FCM is time-consuming and