B. 輸血前後の感染症検査・輸血前検体保管実施状況調査

日本輸血・細胞治療学会、日本臨床衛生検査技師会では、平成16年度から「輸血業務に関する総合的アンケート調査」を行っている。平成16年度～20年度に行われた同調査の中から、輸血前後の感染症検査、輸血前検体保管に関連する事項を抽出し、それらの実施状況や、「輸血療法の実施に関する指針」に書かれている内容の遵守状況などを解析した。なお、同調査は厚生労働科学研究費補助金、「血液新法に伴う輸血管理体制と安全管理・適正使用マネジメントシステムの構築」研究班（主任:髙橋孝喜）、「同種血輸血安全性向上に伴う自己血輸血適応の再検討」研究班（主任:佐川公矯）、「ウイルス肝炎感染防止体制の確立に関する総合研究」研究班（主任:山口一成、浜口功）の援助を得た。

1）アンケート調査の概要

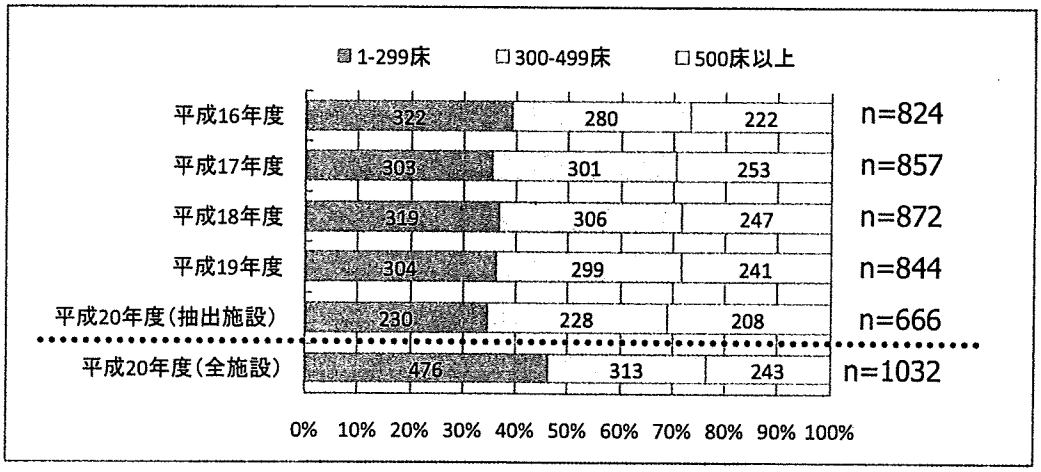
本アンケート調査における輸血前後の感染症検査や輸血前検体保管に関する設問内容は、調査期間（平成16年度～20年度）を通じ原則的に変わっていないが、毎年の集計結果を考慮し設問内容を若干修正している部分がある。平成16年度～18年度までアンケートの対象施設は同一で、アンケートの構成も一律であったが、対象施設の規模や対象施設数の拡充などの理由から、平成19年度以降アンケートを基本調査と詳細調査に分けた。平成19年度の詳細調査の内容は、輸血後感染症検査陽性症例に関する調査のみで、輸血前後の感染症検査・輸血前検体保管に関する項目は基本調査に含まれている。平成20年度には、対象施設数を大幅に増やしたため輸血前後の感染症検査・輸血前検体保管に関する設問のほとんどを詳細設問に移行した。

平成16年度～18年度は、300床以上で血液製剤使用量が3,000単位以上の全医療機関777を含む1,355施設にアンケートを送付し、60パーセント台の回答率であった（表1）。平成19年度は、平成18年度までと同様の施設を対象に調査を行った。その際、全施設を対象にした基本設問と、日本輸血・細胞治療学会認定施設を対象とした詳細設問にわけて調査した。平成20年度は、全国の医療機関に対して実施された血液製剤年間使用量調査と同時に調査を行ったため、基本設問は7857施設、詳細設問は2046施設を対象とした。これら2046施設は平成19年度までの調査対象施設と異なるため、経年変化の解析を目的に平成20年度の調査施設の中から平成19年度に調査を依頼した施設を抽出した。すなわち平成20年度に詳細調査を依頼した2046施設中回答のあった1032施設（50.4%）から、平成19年度にも調査を依頼した施設を抽出したところ666施設が一致した。回答施設数と回答率は表1に示すとおりである。図1には回答施設の病床数分布を示す。アンケート回答施設の病床数別構成比には大きな変動はない。

表 1:アンケート調査の概要

回	年度	調査依頼施設数	回答施設	回答率(%)
1.	平成16年度	1355	829	61.18
2.	平成17年度	1355	857	63.25
3.	平成18年度	1355	872	65.35
4.	平成19年度			
	基本設問	1341	844	62.94
	詳細設問	1341	375	27.96
5.	平成20年度			
	基本設問	7857	3206	40.83
	詳細設問	2046	1032	50.44

図 1:回答施設の規模



2)受血者に対する説明と同意

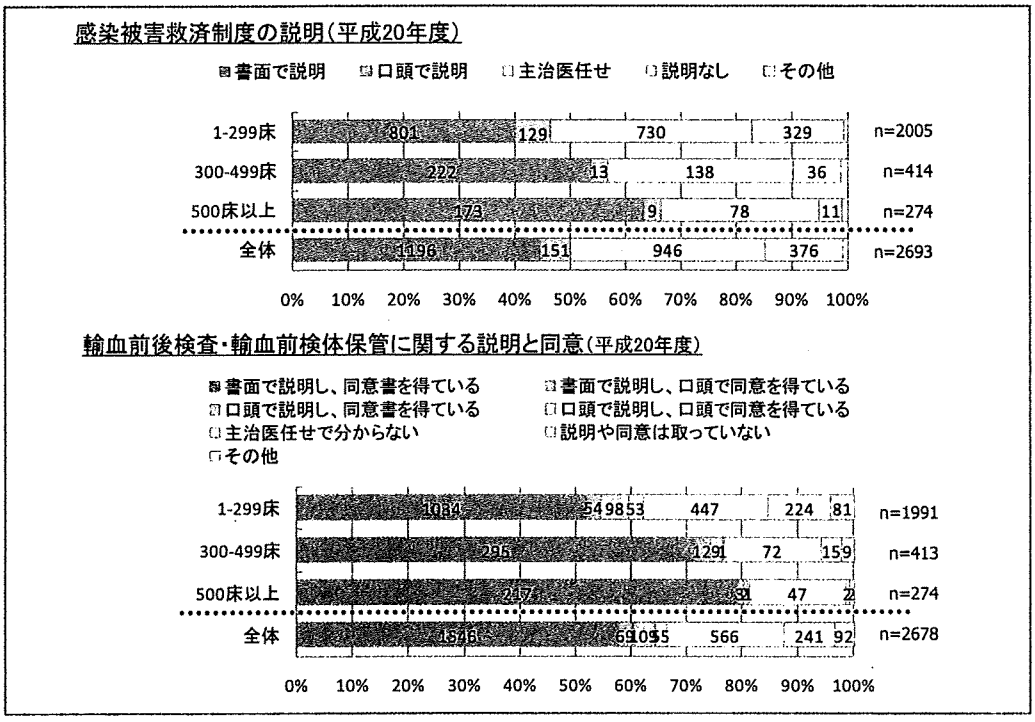
①感染被害救済制度(生物由来製品感染等被害救済制度)についての説明

「輸血療法の実施に関する指針」の輸血療法に関わる説明と同意に必要な 8 項目の中に、副作用・生物由来製品感染等被害救済制度と給付の条件などがあげられている。平成 20 年度調査の基本設問に回答のあった 3206 施設において、感染被害救済制度の説明を書面を用いて行っている施設は 44.4%であった(図 2)。病床数が多い施設ほど感染被害救済制度について書面を用いて説明している割合が多かった。

②輸血前後の感染症検査・輸血前検体保管についての説明

輸血前に、輸血前後の感染症検査や輸血前検体保管について説明し、同意を得ているか尋ねた。全体の57.7%は書面による説明と同意を実施していると回答していた(図2)。感染被害救済制度と同様に、病床数が多い施設ほど書面を用いて説明している割合が多かった。

図 2:感染被害救済制度・輸血前後感染症検査・輸血後感染症検査における説明と同意



3)輸血前感染症検査

①輸血前検査の実施状況

原則としてすべての患者に輸血前検査を実施していると回答した施設の割合は、平成 16 年度 73.1%、17 年度 71.0%、18 年度 72.7%であった(図 3)。平成 19 年度調査から設問内容を一部変更し、遡及調査ガイドラインに準じた感染症マーカー検査が行われているか、また行われていない場合の理由について尋ねた。輸血前検査として独立して原則全例に行っているとの回答は平成 19 年度 22.1%、平成 20 年度 23.3%で、入院時検査や術前検査と合わせて行っているとの回答は平成 19 年度 61.0%、20 年度 59.6%であった(図 4)。輸血前検査を行っていないと回答した施設は平成 19 年度 10.5%、20 年度 9.8%であった。輸血前検査を行っていないと回答した施設のうち、未実施の理由として輸血前検体保管の実施をあげた施設は、それぞれ 72.7%、68.0%であった。

図 3: 輸血前検査実施の有無(平成 16 年度～18 年度)

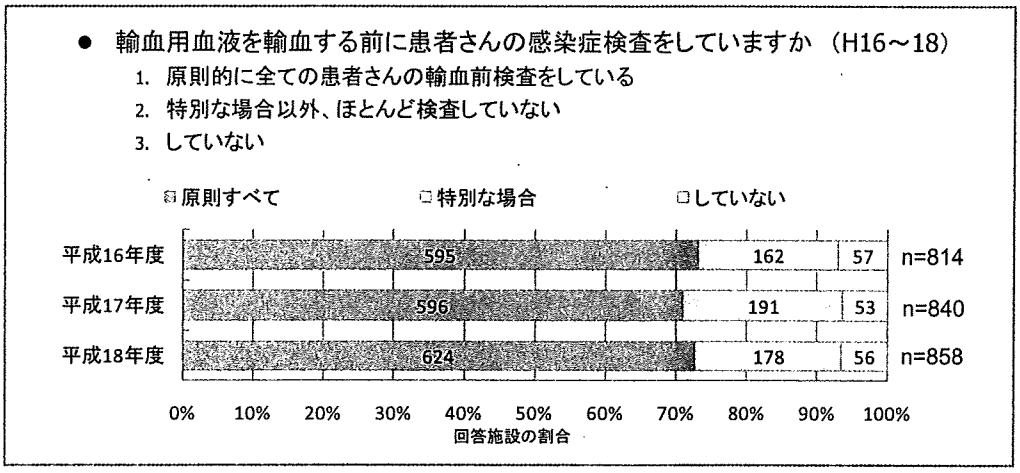
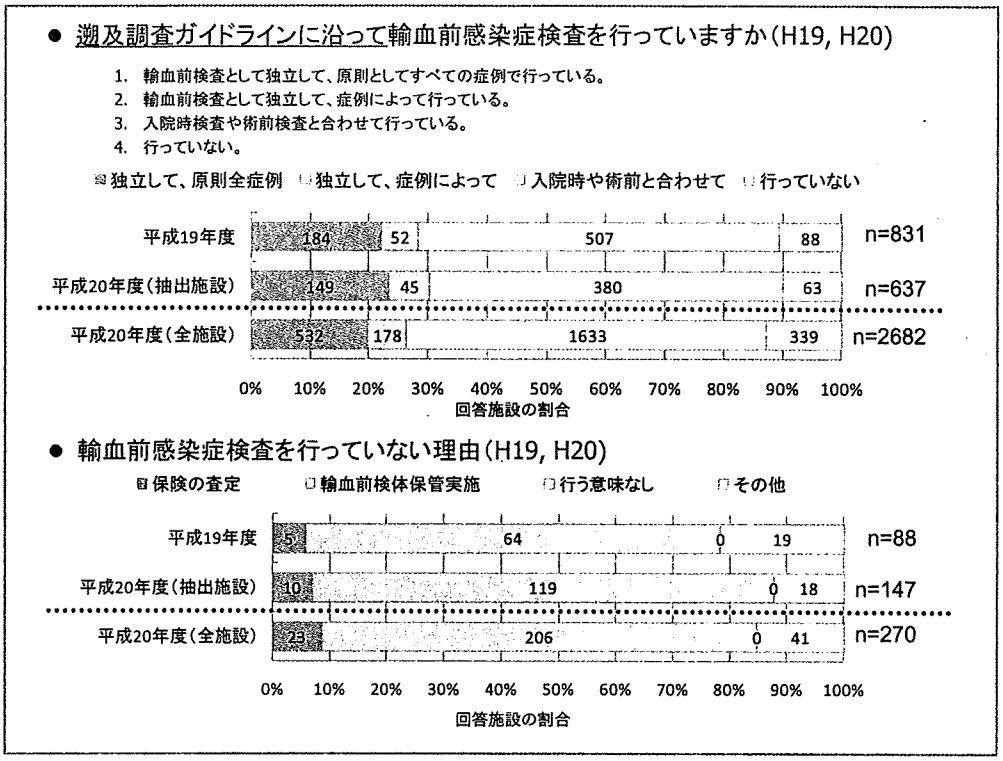


図 4: 輸血前感染症検査実施の有無(平成 19 年度～20 年度)



②輸血前検査に採用している感染症マーカー

「輸血療法の実施に関する指針」には、輸血前検査の感染症マーカーとして、HBs 抗原(HBsAg)、HBs 抗体(HBsAb)、HBc 抗体(HBcAb)、HCV 抗体(HCVAb)、HCV コア抗原(HCVcAg)、HIV 抗体(HIVAb)の 6 種類が記載されている。前項の調査(図 3、図 4)では約90%の施設で輸血前に何らかのウイルスマーカー検査が行われていることが明らかとなった。そこで、本設問では輸血前検査として実際に採用している感染症マーカー項目を尋ねた。調査を開始した平成16年度にはすでにほぼ100%の施設でHBsAg、HCVAbは輸血前検査項目として採用されていた(表 2)。すなわち、従来から入院時検査や術前検査として用いられている HBsAg、HCVAb 検査を輸血前検査として代用している施設が多いものと推定できる。平成 16 年 9 月に一部改正された「輸血療法に実施に関する指針」が周知徹底されるにつれ、HBsAb、HBcAb、HCVcAg の採用率は年々高くなってきたが、平成 20 年度は HBsAb:51.4%、HBcAb:45.3%、HCVcAg:41.6%であった。HIVAb の採用率は、平成 20 年度で 70%であった。

表 2：輸血前検査に採用している感染症マーカー（平成 16 年度～20 年度）

(単位:%)

検査項目	H16年度	H17年度	H18年度	H19年度	H20年度(抽出施設)	H20年度(全施設)
HBs抗原*	99.2	98.7	98.4	99.2	98.0	98.7
HBs抗体*	18.5	32.8	38.8	43.3	51.4	51
HBc抗体*	11.8	26.5	34.1	35.5	45.3	42.6
HBe抗原				0.3	1.3	1.5
HBe抗体				0.1	0.8	1.2
HBV-DNA			0.6	1.2	1.8	1.6
HCV抗体*	98.7	97.7	95.6	98.7	97.5	97.7
HCVコア抗原*		20.9	29.1	32.4	41.6	38.4
HCV-RNA			0.0	0.0	0.8	0.8
HIV抗体*	31.0	44.6	48.4	54.3	56.7	54
HIV抗原･抗体				7.0	12.3	11.6
HTLV-I 抗体	6.2	4.5	4.5			
梅毒	88.4	76.0	68.4			
回答施設数	595	596	624	743	397	713

＊:輸血療法の実施に関する指針の推奨項目

③輸血前検査として実施している感染症マーカーの組み合わせ

平成 20 年度調査における輸血前検査として実施している感染症マーカーの組み合わせを表 3 にまとめた。HBsAg と HCVAb の組み合わせ、すなわち、いわゆる入院時検査に相当する検査の組み合わせで輸血前検査を行っている施設が 44.3%、「輸血療法の実施に関する指針」に従った輸血前検査項目で検査を行っている施設が 38.0%であった。

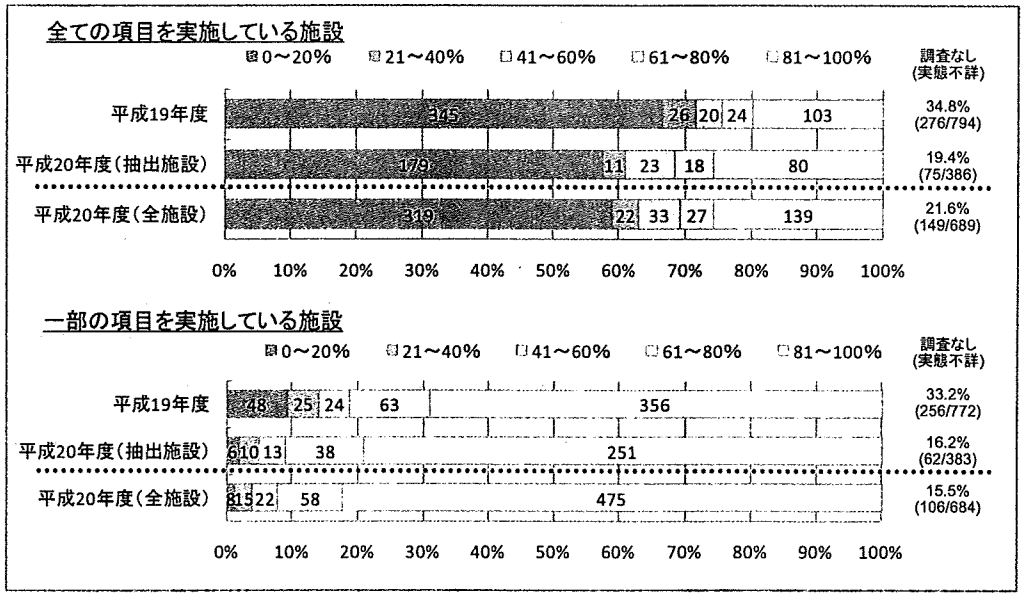
表 3:輸血前検査として実施している感染症マーカーの組み合わせ

HBsAg	HBsAb	HBcAb	HCVAb	HCVcAg	回答数	
●			●		176 (44.3%)	→ いわゆる入院時検査
●	●	●	●	●	151 (38.0%)	→ 輸血前検査
●	●		●		25 (6.3%)	
●	●	●	●		18 (4.5%)	
●	●		●	●	5 (1.3%)	
●		●	●	●	4 (1.0%)	
●		●	●		4 (1.0%)	
●	●	●		●	2 (0.5%)	
●	●				1 (0.3%)	
●			●	●	1 (0.3%)	
●				●	1 (0.3%)	
●					1 (0.3%)	
	●	●	●	●	1 (0.3%)	
	●		●		1 (0.3%)	
			●		1 (0.3%)	
					5 (1.3%)	
389 (98.0%)	204 (51.4%)	180 (45.3%)	387 (97.5%)	165 (41.6%)	397 (100%)	

④受血者を母集団とした輸血前検査実施率

各施設の受血者を母集団とした輸血前検査実施率を、0～20%、21～40%、41～60%、61～80%、81～100%の 5 段階に分け、その実施率を調査した。指針に示されている感染症マーカーすべてを輸血前検査として実施していると回答した施設で、それらの検査が 81%以上の受血者に行われていると回答した施設は、平成 19 年度 19.9%、20 年度 25.7%にすぎなかった(図 5)。逆に、指針に示されている全マーカーの検査実施率が 20%を下回ると回答した施設の割合は、平成 19 年度 66.6%、20 年度 57.6%であった。いずれかの感染症マーカー検査が 81%以上の受血者で実施されていると回答した施設の輸血前検査実施率は、平成 19 年度 69.0%、20 年度 78.2%であった。入院時検査や術前検査として HBsAg、HCVAb 検査が輸血前検査として多くの施設で実施されている現状を反映しているものと考えられる。

図 5:受血者を母集団とした輸血前検査実施率(平成 19 年度、20 年度)

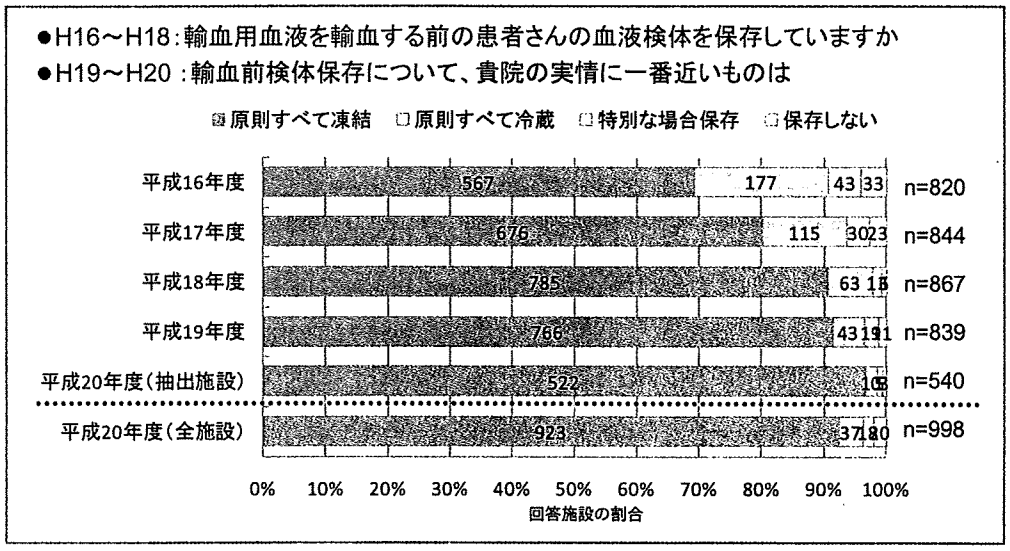


4)輸血前検体保管

①輸血前検体保管の実施状況

輸血前検体保管の実施状況を図 6 に示す。平成 16 年度～18 年度の設問は、“輸血用血液を輸血する前の患者さんの血液検体を保存していますか”、平成 19 年度、20 年度は、“輸血前検体保存について、貴院の実情に一番近いものは”、とした。輸血前検体保管の実施率は、年々増加しており、平成 20 年度には 96.7%の施設で輸血前検体の凍結保管が行われていた。図 6 には、平成 20 年度にアンケート調査を実施した全施設の回答状況も加えてあるが、輸血前検体を凍結して保管している施設は 92.5%であった。

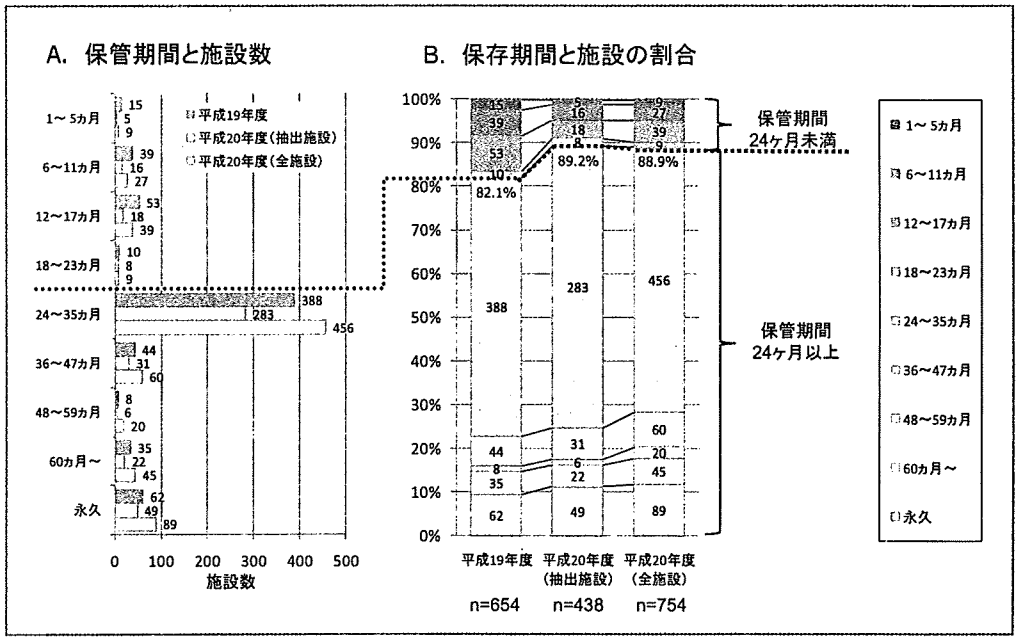
図 6:輸血前検体保管の有無



②輸血前検体の凍結保管期間

平成 19 年度、20 年度の凍結保管期間と回答施設数、その割合を図 7 に示す。輸血前検体を 24 ヵ月以上凍結保管している施設は、平成 19 年度 82.1%、20 年度 89.2%であった。平成 20 年度の全施設での割合は 88.9%であった。

図 7:輸血前検体の保管期間



別紙４

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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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)

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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*.

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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, Super-Script 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

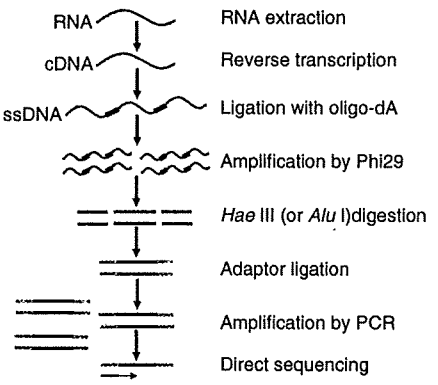

Fig. 1 Overall scheme for RDV ver4.0

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-AAAAAAAAAAAAAAAAAA-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].

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.

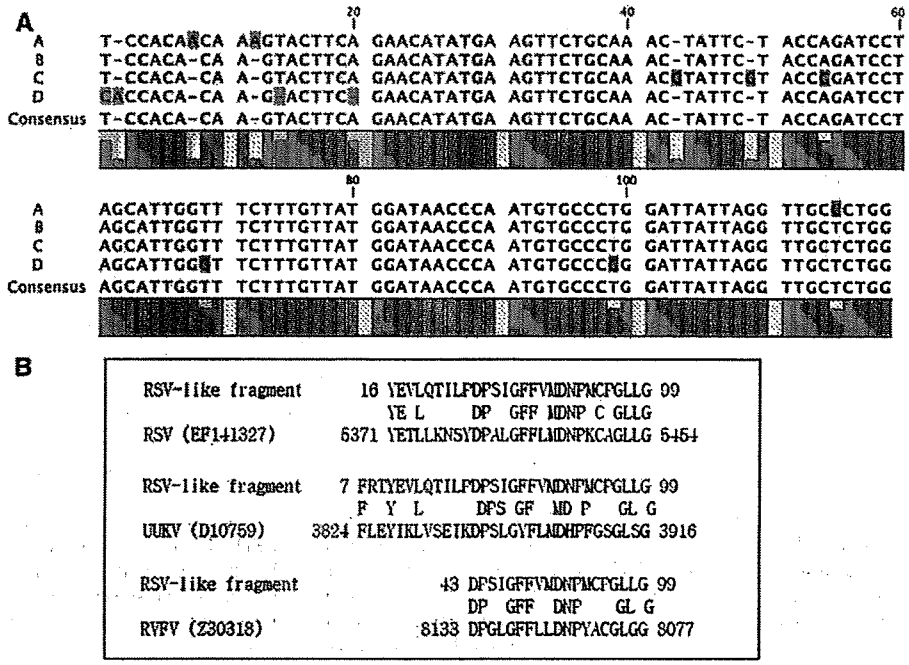

Fig. 2 Rice stripe virus-like sequence obtained using RDV ver4.0. **a** Alignment of four nucleic acid sequences (**a–d**) obtained from number 12 homogenate using RDV ver4.0. **b** Homology of RSV-like sequence to RSV, Uukuniemi virus (UUKV), and Rift Valley fever virus (RVFV)

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/

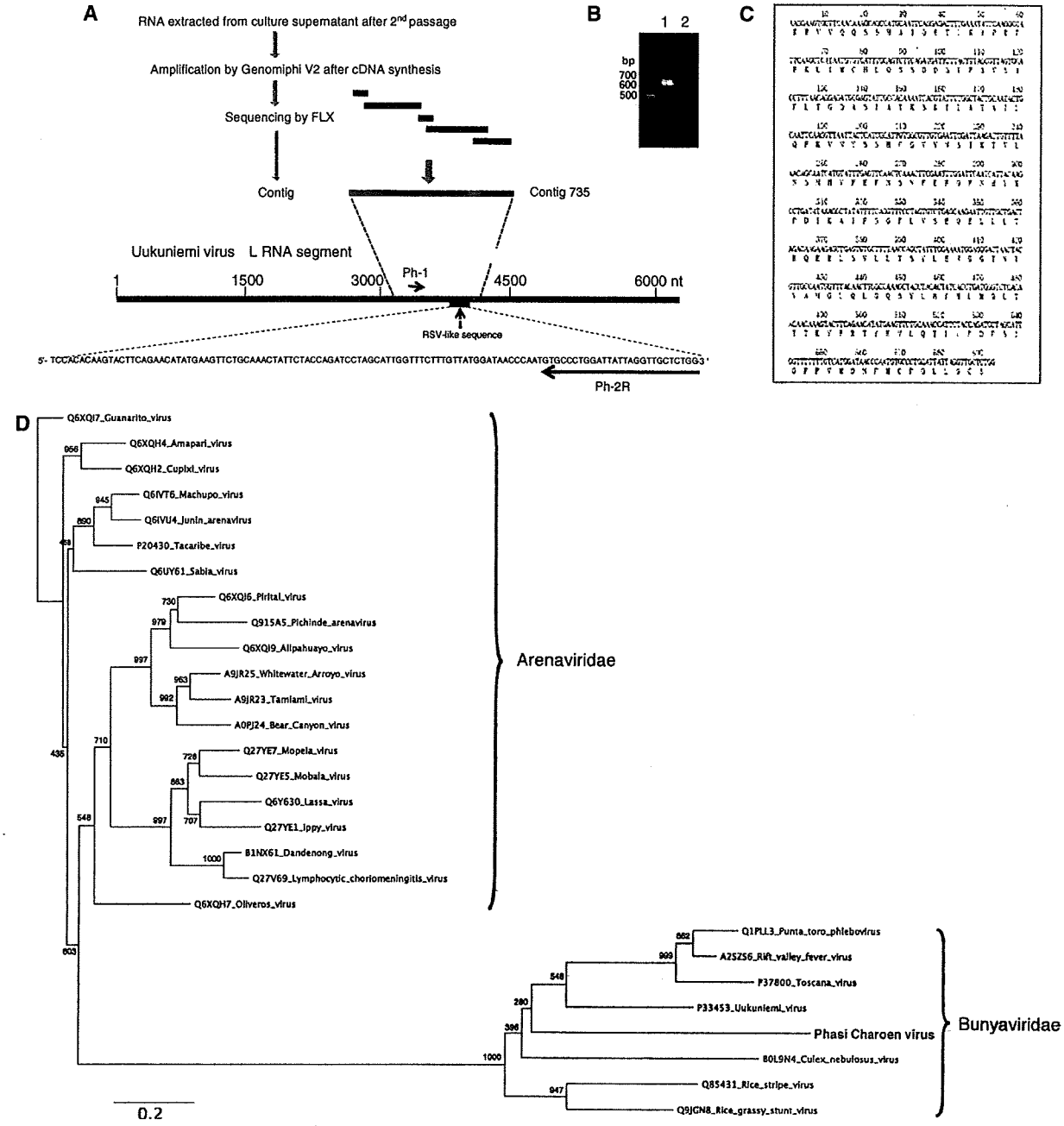

Fig. 3 Phylogenetic tree analysis of PhaV. **a** Contig 735 was located on the UUKV L segment. Primers (Ph-1 and Ph-2R) were designed for amplification within contig 735. **b** cDNA from supernatant of the fourth passage was amplified (lane 1). cDNA from mock-infected supernatant was used as a control (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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Detection of a new bat gammaherpesvirus in the Philippines

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Abstract A new bat herpesvirus was detected in the spleen of an insectivorous bat (*Hipposideros diadema*, family *Hipposideridae*) collected on Panay Island, the Philippines. PCR analyses were performed using COnsensus-DEgenerate Hybrid Oligonucleotide Primers (CODEHOPs) targeting the herpesvirus DNA polymerase (DPOL) gene. Although we obtained PCR products with CODEHOPs, direct sequencing using the primers was not possible because of high degree of degeneracy. Direct sequencing technology developed in our rapid determination system of viral RNA sequences (RDV) was applied in this study, and a partial DPOL nucleotide sequence was determined. In addition, a partial gB gene nucleotide sequence was also determined using the same strategy. We connected the partial gB and DPOL sequences with long-distance PCR, and a 3741-bp nucleotide fragment, including the 3′ part of the gB gene and the 5′ part of the DPOL gene, was finally determined. Phylogenetic analysis showed that the sequence was novel and most similar to those of the subfamily *Gammaherpesvirinae*.

Keywords Bat virus · Direct sequencing · Herpesvirus · Virus discovery · RDV

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With the emergence of zoonotic viruses, including Nipah, Hendra, and Ebola viruses as well as severe acute respiratory syndrome (SARS) coronaviruses, there has been increasing interest in the role of bats as hosts for pathogens. Over 80 viruses have been isolated or detected in bats by nucleic acid analysis [1]. Herpesviruses are widely disseminated in vertebrates, and most mammalian orders have yielded at least one herpesvirus. However, no herpesviruses from bats are listed in the universal virus database [2]. A few herpesviruses were recently molecular characterized in bats [3, 4]. In this study, we examined bats in the Philippines in an attempt to find a new herpesvirus.

Forty-five insectivorous bats, *Miniopterus australis*, family *Vespertilionidae* ($n = 23$), *Miniopterus schreibersii*, family *Vespertilionidae* (17), *Miniopterus tristis*, family *Vespertilionidae* (2), *Rhinolophus arcuatus*, family *Rhinolophidae* (1), *Hipposideros diadema*, family *Hipposideridae* (1), *Myotis macrotarsus*, family *Vespertilionidae* (1), and one frugivorous bat, *Ptenochirus jagori*, family *Pteropodidae*

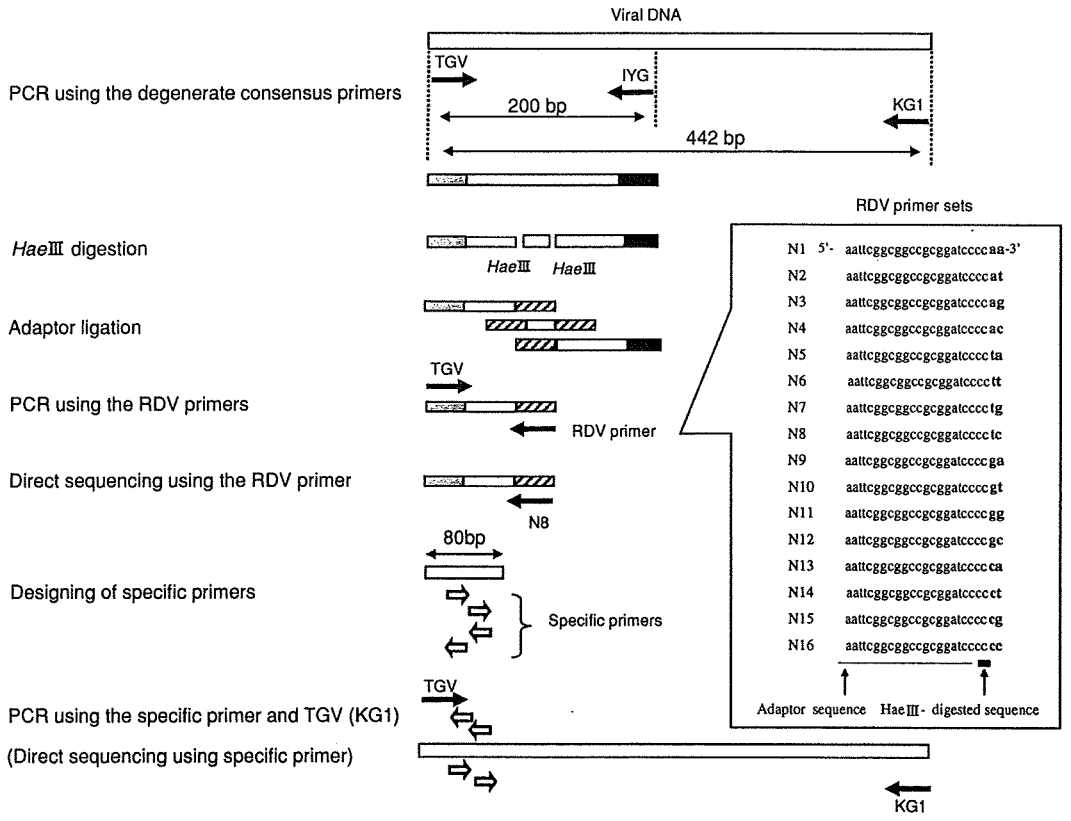

Fig. 1 Overall scheme for direct sequencing with RDV primer sets

(1), were collected at two sites on Panay Island, the Philippines, in 2008. The bats were euthanized under sedation as described previously [5]. Samples of approximately 100 mg of each spleen were used for DNA extraction using a QIAamp DNA mini kit (QIAGEN), according to the manufacturer's instructions. To detect herpesviruses in field samples, we used COnsensus-DEgenerate Hybrid Oligonucleotide Primers (CODEHOPs; nested PCR) for amplification of a partial herpesvirus DNA polymerase (DPOL) gene sequence [6]. These consensus primers are known to be effective for detecting herpesviruses from any vertebrate host. An amplicon of approximately 200 bp of the DPOL gene was obtained in the DNA sample from an insectivorous bat, *H. diadema* (data not shown). Although direct sequencing was performed using CODEHOPs to avoid contamination of DNA in our laboratory, this was difficult due to the high degree of degeneracy of the primers.

Recently, we developed a new method, rapid determination system of viral RNA sequences (RDV), for sequence-independent determination of viral fragment sequences without cloning [7–9]. As described in our previous reports and shown in Fig. 1, the RDV method includes direct sequencing technology. Each RDV primer contains the adaptor sequence, 4 nucleotides including CC (the end of the sequence after *Hae*III digestion) and 2 variable nucleotides. After purification of the 200-bp PCR product from the gel, DNA was digested with *Hae*III, and subjected to adaptor ligation as described previously [9]. PCR was performed using the RDV N1 to N16 primers and the degenerate TGV or IYG primer. We expected selective amplification of the templates having the RDV primer sequence and TGV (or IYG) primer at each end. PCR products were electrophoresed on agarose gels, and a DNA band of approximately 80 bp was obtained when the TGV and RDV N8 primers were used for amplification (data not shown). The DNA fragment was extracted from the gel, and direct sequencing was performed using the RDV N8 primer. Based on the fragment sequence obtained by direct sequencing, several specific primers were then newly designed. PCR was performed using these specific primers and the KG1 primer. The resultant PCR products were subjected to direct sequencing again. A 442-bp DPOL nucleotide fragment, corresponding to the region of amplification product with TGV and KG1, was determined (DDBJ accession no. AB459535).

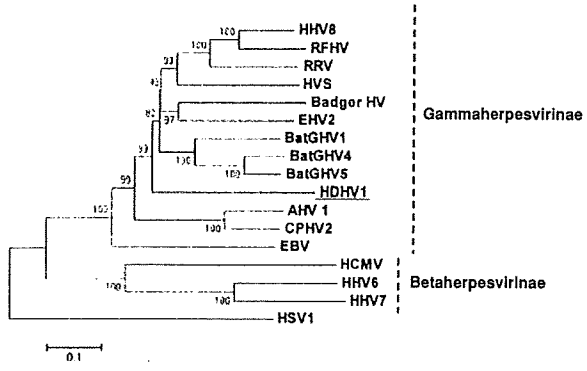

Fig. 2 A phylogenetic tree was constructed using a multiple alignment of 914 aa, consisting of concatenated gB and DPOL amino acid sequences. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. Phylogenetic analyses were conducted in MEGA4 [11]. The tree was rooted to herpes simplex virus type 1 (HSV1) (X14112). The evolutionary distances were computed using the Poisson correction method and are given in units of the number of amino acid substitutions per site. All positions containing alignment gaps and missing data were eliminated from the dataset. The herpesviruses used for comparison and their accession numbers are as follows: alcelaphine herpesvirus 1 (AHV1), NC_002531; badger herpesvirus (BadgerHV), AF376034; bat gammaherpesvirus 1 (BatGHV1), DQ788623; BatGHV4, DQ788627; BatGHV5, DQ788629; caprine herpesvirus 2 (CPHV2), AF283477; Epstein-Barr virus 1 (EBV)(human herpesvirus 4), NC_007605; equine herpesvirus 2 (EHV2), NC_001650; human cytomegalovirus (HCMV), NC_006273; human herpesvirus 6 (HHV6), AF157706; HHV7, NC_001716; HHV8 (Kaposi's sarcoma virus), NC_003409; retroperitoneal fibromatosis-associated herpesvirus (RFHV), AF005479; rhesus monkey rhadinovirus (RRV), AF083501; saimiriine herpesvirus 2 (HVS), NC_001350

BLAST search suggested that the DPOL sequence was novel and most similar to those of gammaherpesviruses. Therefore, PCR was performed with another nested primer set targeting the gB genes of gammaherpesviruses [4]. First PCR was performed using RH-gB 1s and RH-gB 1as primers. After second PCR with RH-gB 2s and RH-gB 2as primers, an amplicon of approximately 450 bp of gB gene was obtained. To determine the nucleotide sequence of partial gB genes using direct sequencing, the same strategy used for determination of the partial DPOL sequence was applied. As a result, a 631-bp gB nucleotide fragment, corresponding to the region of amplification product with RH-gB 1s and RH-gB 2as primers, was determined. Then, we connected the partial gB and DPOL sequences with long-distance PCR, using specific primers, which were designed based on the obtained sequences in gB and DPOL regions. A 3741-bp nucleotide fragment including the 3′ part of the gB gene and the 5′ part of the DPOL gene was finally determined and deposited in Genbank (DDBJ accession no. AB490083). In tblastn search, it was demonstrated that concatenated gB and DPOL deduced amino acid sequence (1146 aa) was novel and most similar to those of retroperitoneal fibromatosis-associated herpesvirus (58% amino acid sequence identity). We have tentatively named this virus "Hipposideros diadema herpesvirus 1 (HDHV1)." A phylogenetic tree was constructed using the neighbor-joining method with concatenated gB and DPOL deduced amino acid sequence (gB; 304 aa, DPOL; 610 aa) and the available sequences of known herpesviruses (Fig. 2). The tree confirmed that HDHV1 belongs to the *Gammaherpesvirinae* and suggested that HDHV1 is not assigned to the known genus. The tree also showed that HDHV1 is not placed in the same group with the known bat gammaherpesviruses. However, further characterization of HDHV1 is needed to reveal its taxonomic assignment.

Recently, bats have been described as hosts for herpesviruses in several countries in Europe, South America, and Asia [3, 4]. This report shows the detection of a new gammaherpesvirus in the Philippines, and confirms the wide geographical distribution of herpesviruses in bats. As bats display a high degree of diversity and account for 20% of the approximately 4,800 mammalian species [10], these animals are potential hosts for many unknown herpesviruses.

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