輸血前後の感染症検査についての意見　（平成19年度）　別表-6

番号	事項
46	厚労省の推奨する輸血前後の感染症検査項目の根拠が不明瞭。コスト・エフェクティブネスを無視しており、メーカや検査業界の（癒着）圧力を感じる！
47	厚労省の推奨項目は、現場の実情を考えるとかなり無理があるように感じます。
48	厚労省の推奨項目全てを院内で行うことは困難な状況特に核酸増幅検査やHIV検査などは問題が多いと思う
49	厚労省の推奨事項より、輸血学会のガイドラインの方が現場に則しているので統一して進めていっていただきたい。厚労省は推奨するだけでなく保険適応になることにも働きかけていただきたい。でなければ、実施率のアップは望めないと思う。
50	高齢者の延命のための輸血が多い。実際は、医師の判断で行われないことが多い。完璧に全員行うことは、難しい。
51	国による大幅な財政援助がないと広がらない
52	今回の集計では、死亡している患者は対象外ということで、総数（分母）には入れない。また転医先に依頼した場合は確実に実施し、結果がわかった患者のみ実施数に入れた。
53	今後システムを作成するが、体制つくりや、患者への通知など負担が大きい
54	今後実施予定。実施率が気になるところである。
55	査定が無いようにしてほしい
56	査定されないことがまず１番。また定期的に輸血している疾患（血液疾患）の場合，どのくらいの間隔で検査をするのが望ましいのかが，解決されるべきである
57	査定されることがあるので、保険機構に必要な検査で査定しないでほしいと通知してほしい。
58	事務仕事が増えすぎる。H19・7から実施した。
59	次年度開始予定。頻回輸血の患者様への対応を思案しています。
60	実施分、保険請求がとおることを希望する。
61	社会保険で査定はないが、国民保険は疾患名が無い為査定されるので輸血実施した場合査定しないで欲しい。
62	主治医の考えに従う部分あり。臨床を見て医師に指示を。
63	受検率がなかなか上がらないこと。
64	書面で輸血感染症検査をすすめても受検する人がいないのは、医師の説明の方法によると思います。
65	小児（特に新生児）は採血が出来ないため輸血前検体保存が難しく苦慮している。
66	小児の輸血前検体確保が難しい転院された患者様の受診率が把握できない
67	小児を対象としているため、採血量の制約があり、輸血前後の検査は最低限となっている。保存する検体量も充分といえない状況である。いかにすべきか悩んでいる。
68	小児病院なので、新生児～小児の感染症検査の推奨項目を輸血前後実施するのは採血量の面からもかなり大変です。
69	推奨項目数が多く現実的ではない。輸血後検査は何か問題があったときに行い、通常は前検体保存で対応。
70	生保も国保も保険請求した場合取れると文章や口頭で言っているにもかかわらず査定してくるときがあり再審査を請求しても復活しないときがある。　国として一貫した取り決めを望む
71	前の検査はコストが取れないので、病院としてはできない。
72	前検査の遺伝子レベルでの実施は無駄である。しかし、専用容器で別採血をして保管するには経費も場所も必要であり、多くの施設で実施できるとは思えない。
73	前後に検査を行うのが理想的であるとは思うが、なかなか実際には困難である。検体保管をしっかりすることでカバーするのが現実的と考える。
74	前後の感染症検査は保健請求できるようにしてほしい
75	前後の検査結果の把握など、検査部側から臨床に通知等を行わないいけない。期間が空くため。
76	前後実施の実施率をすぐに割り出すシステムがないので現状把握等が難しい
77	全ての項目が保険請求されると良い
78	全て実施するよう現在準備中
79	全症例の保険適応を望む。
80	全輸血患者の輸血前患者検体保管が最も現実的である。なお、保管に対し保管料を認める等も必要である。
81	都道府県で保険請求出来たり出来なかったりばらつきがあるから、統一して欲しい。
82	当院では高齢の輸血患者が多く輸血後感染症検査の受検率の上昇は望めそうにない。B型C型肝炎ウィルス陰性輸血患者は総数の３０％である。
83	当院では自前の検査室を持ち、かなり感度のいい試薬を使用しているので特にＤＮＡやコア抗原まで調べなくても、院内検査で対応できると考えています。当日検査・結果説明が患者様にとっていいことと思います。
84	当院では輸血後感染症検査の徹底は平成19年11月からなので輸血後検査はまだ出ていない現状です。
85	当院で輸血前後の感染症検査を行う事を考えたが医事科より神奈川県は保険が通らないから出来ないと言われ今日に至る。自治体により考え方が違いすぎる。
86	当院に受診予定がない、あるいは輸血を実施した診療科とは違う科に受診する等の患者様のワークフローの作成や報告ルートの確立が困難であった。社会保険庁の保険請求の回答が不明確、又たらい回しである。
87	当院の検査費用は全て病院負担の為検体全てを凍結保存とし疑わしき事例の場合の検査のみで良いと考えている
88	当院の現状は輸血前後のセットは組んであるが、オーダは医師任せで輸血前検体のみ凍結保存している。
89	当院は平均入院日数9.8日で退院後はかかりつけ医に患者を返すので開業医の先生方のご協力が必要と考える
90	当院規模の病院だと、技師の教育・啓蒙から必要な場合があるので、困難である。保険制度にメリットのある条件があれば良いと思います。当院は、ｱﾙﾌﾞﾐﾝ製剤の使用に医師の理解が得られず、管理料Ⅱも困難な状況です。
91	特に輸血前検査を実施した場合保険で査定しないで欲しいと思う
92	日本輸血・細胞治療学会の運用マニアルを基本にするのがよいと思います。
93	必ず保険請求できるようにしていただけたらと思います。
94	病名疑いの記載がなくても、一定の条件（手術や輸血の事実）あれば査定すべきではない
95	頻回輸血されている患者様の輸血後感染症は、いつ行ったらいいのか？
96	頻回輸血される血液疾患の患者につき、輸血前検査後、輸血を実施しなかった場合であっても、輸血前感染症検査の保険請求を認めてはどうか

輸血前後の感染症検査についての意見　（平成19年度）　別表-6

番号	事項
97	頻回輸血患者の検査項目の選択、検査の間隔がよくわからない。感染リスクと患者負担のバランスをどのように捕らえたらよいのか悩みます。
98	頻回輸血患者の検査実施時期について苦慮している
99	頻回輸血患者への対応が不明である。指針を示してもらいたい。
100	保険が認められなければ推進は難しい。
101	保険で確実に認めてほしい（特にＨＩＶ）
102	保険で査定されないことがはっきりすれば普及すると思う
103	保険で査定されないことが必要不可欠。輸血前は、術前検査としての感染症検査でよいのではないでしょうか。
104	保険で査定されない様にして欲しい。
105	保険で認められないので医師にすすめにくい。実費では患者に負担が大きい
106	保険の査定の有無は、地域、査定者によって差があるのが現状であり、改善して欲しい。
107	保険査定の可否において都道府県格差が発生していることを解消する必要が有ると考えます
108	保険請求が可能か不可能か不明確。
109	保険請求して査定されないならば，輸血後検査時期を検査室からお知らせして，積極的に輸血後検査を薦めていきたい。
110	法律にしてください。
111	末期患者を持つ医師の協力が得にくい。
112	未開封、ＰＣＲ用前検体があれば、３ヶ月の受検がなくても輸血後は発生時に検査する。患者の自己管理を促す。輸血したことを書面で残し、患者に渡す→本人が希望する医療機関でよい。現在の血液はかなり安全。すべての輸血についてもれなくスクリーニングするのはムダが多い。
113	問題もあると思いますが保険の査定があると思うと検査は出しにくい。
114	輸血に対する保険請求行為１回なのだから輸血前の血液はとりあえず保存しておき、輸血後陽性であれば、戻って検査すればよい
115	輸血を頻回に行う場合の検査時期の基準は直近の輸血から３ヶ月以上あいた場合でよいのでしょうか？
116	輸血後の感染症を検査し、輸血前検査は入院時感染症で代用が合理的と考えるが、輸血後の検査率を上げるのが困難。
117	輸血後の感染症検査の重要性を行政がもっとアピールすべき
118	輸血後の感染症検査は、今年度末に実施率調査を行う予定。輸血前検査は保険が通らない為、検体保存のみ行っている。輸血後は請求時輸血後とコメントを入れるよう改善中。
119	輸血後の感染症検査を厚労省の推奨項目全てをセットで実施した場合保険請求が通るでしょうか。現にＨＩＶは通らないと認識している。
120	輸血後の感染症検査実施率向上のためには医師への周知が必要であるが、周知を徹底できない。
121	輸血後の検査は様々な状況があり、全てを実施するには、システムの整備が必要と考える。
122	輸血後の検査を実施するのは、難しい（忘れてしまう）
123	輸血後感染症検査において、3ヶ月を少しでも経過したものについては適用にならないというのはかなりきびしいのではと思う
124	輸血後感染症検査にて陽性の場合のみ、輸血前保存検体で検査をし、輸血による感染かどうかを判断する。
125	輸血後感染症検査の案内をしているが、受診しない方が多い。
126	輸血後感染症検査は、厚生労働省で義務化し保険点数も取れる形にならないとなかなか困難だと思います。
127	輸血後感染症検査は患者本人にまかせており、すべて実施するには無理があるように思われる。
128	輸血後感染症受検については、多種考慮する条件があり、現状、徹底実施することは難しい状況と思います。
129	輸血後感染症被害を受ける確率がすごく低い現状では、全員に細かい輸血前検査を実施することは、コスト面、医療費面、地球温暖化促進の面から、無駄以上のものと考える。
130	輸血後検査については、そのためだけに来院してもらえることが難しく、実施率が低くなってしまう
131	輸血後検査について輸血を施行した患者のリストを主治医に提出しようと提案したが、Ｄｒが日常の忙しい中、患者に連絡を取るのは難しいと言われ、現在保留中
132	輸血後検査の実施は患者への連絡、検査実施の承諾等正直難しい
133	輸血後検査の実施率は亡くなっていることが多いです。実施率に計算するのでしょうか？　他施設で輸血したが、検査の説明はなくて、当院で後だけ実施ということもあるので普及を。最初はよく査定されました
134	輸血後検査は日本輸血細胞治療学会のマニュアルの項目に変更することにした。
135	輸血後検査をすべての患者に実施することは難しい。転院や外来で、受診しなくなるケースが多い。
136	輸血後半年以内ででもいいのではないか
137	輸血実施後３か月以降に、オーダー画面に注意表示されるシステムがあれば後の依頼漏れは少なくなる。
138	輸血責任医師（輸血療法委員会長）が感染症検査実施に消極的で、検体保存の方向で行っている
139	輸血前と輸血後の感染症検査の保険適応を全国に徹底してほしい
140	輸血前に推薦項目を行うのは、現状無理で、輸血後も肝炎などの症状がなければやらないという医師の意見でした。
141	輸血前の感染症検査を実施しておらず、かつ、検体の保存もない場合救済制度の対象とならないのか教えてください。生物由来感染症等被害救済制度について医薬品医療機器総合機構に質問を出しましたが、返事がありません。
142	輸血前の検査は比較的問題は無いが、輸血後の検査の場合、全症例行なうことは管理上、非常に難しい。
143	輸血前の検体保管が輸血管理料とは別に保険請求ができればよい。
144	輸血前の保存用検体として開栓していないものが必要でしょうか？
145	輸血前はＴ＆Ｓ対応のＯＰＥなど実際には輸血をしない場合も多くあり、検査を行なうタイミング等を考えると実施が難しく、全患者に対して行なう必要はないと考えます。
146	輸血前は検体保存で充分だと思うがコストがかかる。輸血後は検査時期の範囲（保険適用範囲）を明確にしてほしい。
147	輸血前は前検査項目が実施できないのであれば、検体保管しておけば良いのでは。

輸血前後の感染症検査についての意見　（平成19年度）　別表-6

番号	事項
148	輸血前感染症は、入院時と併用し医者の判断で検査し交差試験j時かならず検体保存は行う。輸血後も医者の判断で検査を定期的に行うようにする。陽性時精査する。
149	輸血前感染症検査とその検体の保存だけでは不十分なのでしょうか？
150	輸血前感染症検査の推奨項目が保険で確実に認められるようにしていただきたい。
151	輸血前検査でHIV検査を行うと混合診療になるのではないか
152	輸血前検査のみでよいのでは必要がある場合のみ行うと良いと思う
153	輸血前検査はH19.4月より、輸血後検査はH19.7月より実施。
154	輸血前検査は査定を受けるので実施できない
155	輸血前検体をすべて保存しておけば輸血後に陽性になった検体のみ精査することで問題ないのでは・・・
156	輸血前検体保管は重要と考え実施している。HBV-DNA核酸増幅検査を行うのは非現実的であり、対応を取れなかった。最近学会から出された検査項目であれば実施に向けて取り組みたいと考えている。
157	輸血前検体保存にかかわる費用を医療機関が全て負担しなければならないのはおかしい。輸血管理料に条件が加えられるべき。
158	輸血前検体保存をしていても、輸血前感染症検査を実施する理由があるかどうかについて知りたい。
159	輸血前検体保存を行い、輸血後感染症検査を行っていればいいのではないか。輸血前に多くの検査を行うのは医療費の無駄使いではないでしょうか！
160	輸血前後の感染症の実施には、各医療機関の努力ではなく、義務化するべきだと思う。全国統一のシステム化を希望する。感染症マーカーの絞込み。
161	輸血前後の感染症検査で、ＴＰＬＡとＨＴＬＶ－１を追加したらどうでしょう。
162	輸血前後の感染症検査の広報（なぜ必要かを）を判りやすく記載したものを作成して欲しい。
163	輸血前後の感染症検査を行っていても供血者の検査が受動的であるため、実際に感染していてもほとんどが証明できないことが多い。
164	輸血前後の感染症検査項目は厚生労働省推奨項目に変えることが決定し取り組んでいます。
165	輸血前後の検査は行わず、検体保存だけでは問題ありますでしょうか？
166	輸血前後感染症検査に関しては早急に実施に向けて検討中です。輸血前はT&S、自己血等については、苦慮しています。
167	輸血療法委員会で議題とし、必要性を訴えてきたが、なかなか出してもらえない。子ども病院とはいえ、採血量を期待できる場合もある。電子カルテのオーダー画面を工夫して今後も促して行こうと思っています。
168	輸血療法委員会を通じて検査の重要性は話をしているところであるが、今現在のところ取り組んでくれている医師はいない。また頻回輸血患者等が多くいる当院では、タイミング等を難しく感じている医師もいると思われる。同意書を作り変えて促進する話は１年以上前から出ているが、実際のところなかなか進んでいない状況です。
169	陽転した症例の精査を血液センターに依頼しているが、調査内容がまちまち。内容を固定してほしい。公的な第３者機関による調査のほうが良いのでは？
170	連続して輸血している患者の検査時期がわからない。

【2-050-2】輸血前後感染症検査の問題点や改善点(詳細) 平成20年度 別表-7

番号	回答
1	2-45の回答は(1)・(6)で対応しています。
2	DPCのまるめ項目からは除外して欲しい
3	HIVなど、当院で検査を行っていない為、外注検査となる。
4	HIV抗体検査は同意書を取る必要があること。
5	Q&Aに医師が必要と認める場合であって必ずしも全例に行う必要はないと言う文言がある為実施率が低い
6	スタッフ・患者の負担が増えることに理解を得られる自信がない。
7	一般の方へのマスメディアを使用した広報が効果的であり必要と考えます。
8	院内の機器では対応できないため、設備投資が必要
9	患者からの同意が得られない(特にHIV検査)
10	患者さん、あるいは一般市民の理解
11	患者への説明が困難。
12	感染症検査項目ははじめから核酸増幅検査ではなく、通常の感染疑いの様に血清学的な検査から順番に精度を高める項目の実施で十分と考える。
13	検体の保管場所が確保できない
14	検体保存に関わる費用が医療機関の負担となっている。(輸血管理料が算定できないため。)
15	厚生労働省の勧告であるが、DPC包括されてしまうため、改善を求めます。
16	厚労省より輸血療法の実施に係る指針等に、輸血前後感染症検査は、必須検査と改正すべきである。
17	早期死亡患者が多い。
18	当院は療養型病棟なので、輸血前後の感染症検査は診療報酬の算定ができない。
19	当院包括医療請求のため困難。
20	保管検体の場所、冷凍庫設置など
21	保健所等で無料にて検査を実施できるような体制の整備
22	保険適応にはなるが、実質患者様負担なので　無料化するべきである
23	陽性率が極めて低いのに手間がかかる検査体制を運用することに疑問
24	療養病床では検査項目もの算定ができない為輸血目的の場合は算定可能とできないか?
25	冷凍庫の確保が困難
26	連絡しても来院されない

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
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 ,	Novel virus discovery in field-collected mosquito larvae using an improved system for rapid determination of viral RNA sequences (RDV ver4.0)	Archives of Virology	154	153–158	2009
Shumpei Watanabe, Naoya Ueda, Koichiro Iha, Joseph S. Masangkay, Hikaru Fujii, Phillip Alviola, Tetsuya Mizutani, Ken Maeda Daisuke Yamane, Azab Walid, Kentaro Kato, Shigeru Kyuwa, ukinobu Tohya, Yasuhiro Yoshikawa, Hiroomi Akashi	Detection of a new bat gammaherpes virus in the Philippines	Virus Genes	39	90–93	2009

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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

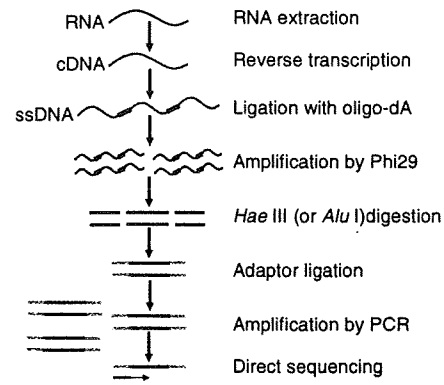

Fig. 1 Overall scheme for RDV ver4.0

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.

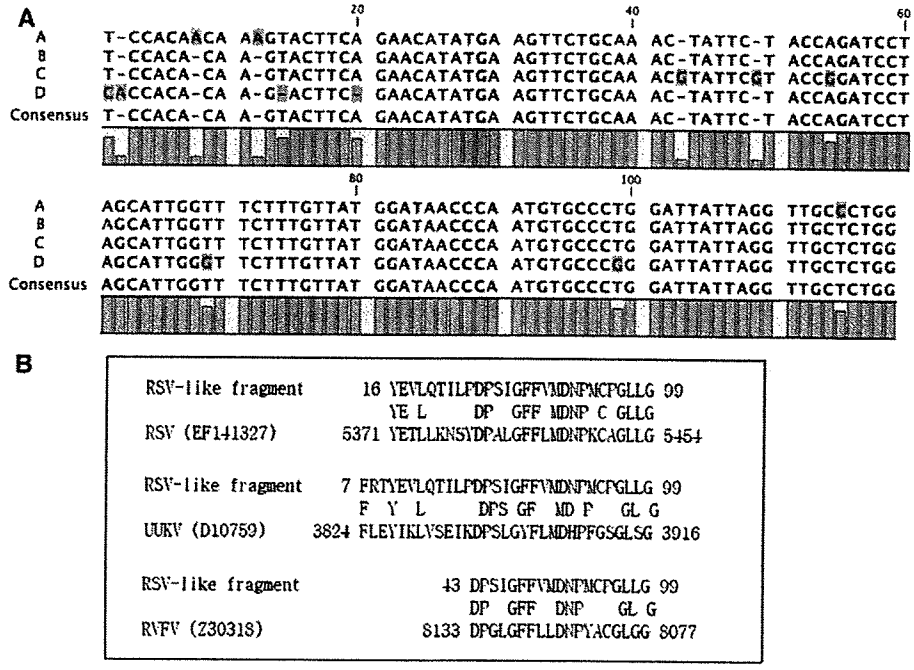

Fig. 2 Rice stripe virus-like sequence obtained using RDV ver4.0. **a** Alignment of four nucleic acid sequences (**a–d**) obtained from number 12 homogenate using RDV ver4.0. **b** Homology of RSV-like sequence to RSV, Uukuniemi virus (UUKV), and Rift Valley fever virus (RVFV)

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/

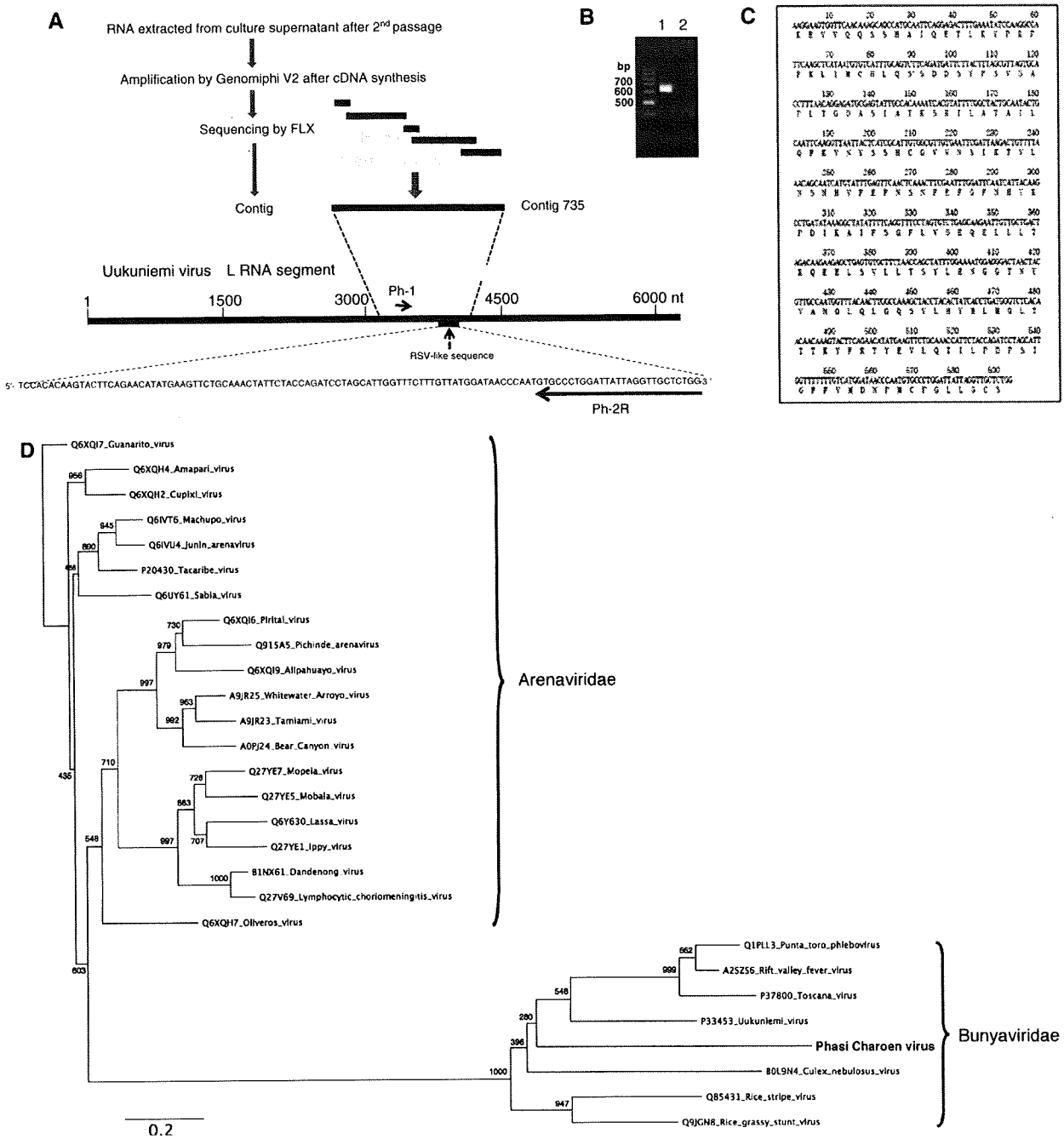

Fig. 3 Phylogenetic tree analysis of PhaV. **a** Contig 735 was located on the UUKV L segment. Primers (Ph-1 and Ph-2R) were designed for amplification within contig 735. **b** cDNA from supernatant of the fourth passage was amplified (lane 1). cDNA from mock-infected supernatant was used as a control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

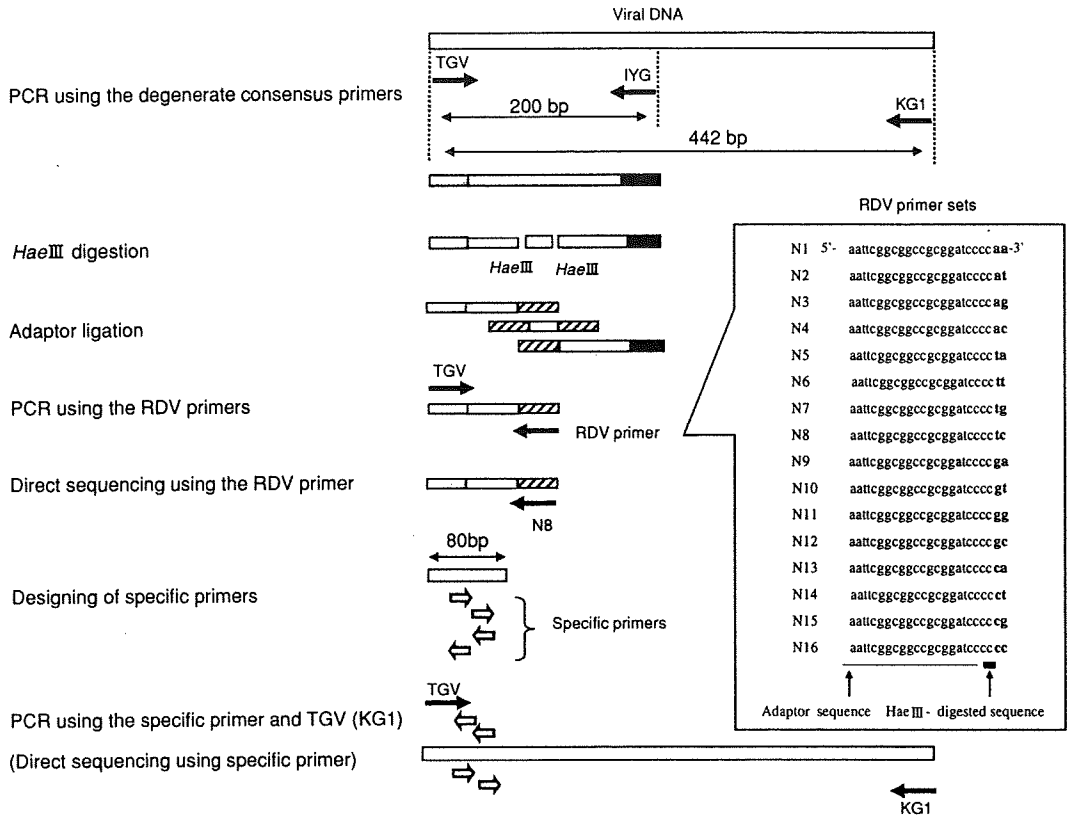

Fig. 1 Overall scheme for direct sequencing with RDV primer sets

(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).

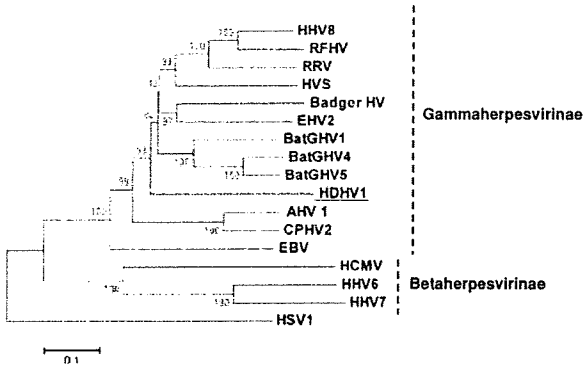

Fig. 2 A phylogenetic tree was constructed using a multiple alignment of 914 aa, consisting of concatenated gB and DPOL amino acid sequences. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The percentages of replicate trees 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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