平成20年度 厚生労働科学研究費補助金輸血関連研究班
第一回合同班会議

輸血医療と血液製剤の安全性向上に向けた総合研究

2008

日時：平成20年7月19日(土) 13:00~18:00
場所：国立感染症研究所 戸山庁舎(2F 共用第一会議室)
〒162-8640 東京都新宿区戸山1-23-1 Tel. 03-5285-1111

○ 挨 拶 13:00~13:10 秋野 公造（厚生労働省 医薬食品局 血液対策課）

○ 演 題 13:10~14:40 ◆座長：高本 滋（愛知医大病院）

1. 日本における血液製剤の副作用サーベイランス体制の確立に関する研究 浅口 功（国立感染症研究所）
2. ヘモビジランスのための病院内輸血副作用監視体制に関する研究 藤井 康彦（山口大医病院）
3. 院内血液製剤の適正な製造体制順守基準に関する研究 大戸 斉（順天堂医大病院）
4. 献血者の安全確保対策に配慮した採血基準の拡大に関する研究 河原 和夫（東京医科歯科大院）
5. 大量出血時における止血凝固の評価と輸血療法に関する研究 高松 純樹（名古屋大医病院）
（山本 晃士）

○ 演 題 14:40~15:50 ◆座長：佐川 公矯（久留米大医病院）

6. 献血者の増加に資する教育教材の開発とその効果の検証 田久 浩志（中部学院大）
7. 第VIII、第IX因子製剤のインヒビター発生頻度に関する研究 吉岡 堯（奈良県立医科大）
8. 献血血液の安全性確保と安定供給のための新興感染症等に対する検査・スクリーニング法等の開発と献血制限に関する研究 倉根 一郎（国立感染症研究所）
（岡田 義昭）
9. 輸血副作用の術前遺伝子ハプトグロビン欠失アリルの迅速簡便な診断法の確立と輸血前診療への臨床応用 神田 芳郎（久留米大学医学部）

○ 休 憩 20分

○ 血液製剤の不活化をめぐるワークショップ 16:10~18:00 ◆座長：山口 一成（国立感染症研究所）

10. 病原体不活化導入に向けた提案 下平 滋隆（信州大学病院）
11. 国際輸血学会（ISBT）報告・不活化 大坪 寛子（国立感染症研究所）
12. ヨーロッパにおける血液製剤の不活化（ヨーロッパ視察報告） 大戸 斉（日本輸血・細胞治療学会）

■問い合わせ先

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別紙４

研究成果の刊行に関する一覧表レイアウト

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

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発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Sakai K, Ueno Y, Ueda S, Yada K, Fukushi S, Saijo M, Kurane I, Mutoh K, Yoshioka K, Nakamura M, Takehara K, Morikawa S, Mizutani T	Novel reovirus isolation from an Ostrich (Struthio camelus) in Japan.	Vet. Microbiol.	134	227-232	2009

Saijo M, Ami Y, Suzaki Y, Nagata N, Iwata N, Hasegawa H, Ogata M, Fukushi S, Mizutani T, Iizuka I, Sakai K, Sata T, Kurata T, Kurane I, Morikawa S.	Diagnosis and assessment of monkeypox virus (MPXV) infection by quantitative PCR assay: differentiation of Congo Basin and West African MPXV strains.	Jpn. J. Infect Dis.	61	140-142	2008
Nakamura S, Yang CS, Sakon N, Ueda M, Tougan T, Yamashita A, Goto N, Takahashi K, Yasunaga T, Ikuta K, Mizutani T, Okamoto Y, Tagami M, Morita R, Maeda N, Kawai J, Hayashizaki Y, Nagai Y, Horii T, Iida T, Nakaya T	Direct metagenomic detection of viral pathogens in nasal and fecal specimens using an unbiased high-throughput sequencing approach.	PLoS ONE	4	e4219	2009

Watanabe S, Mizutani T, Sakai K, Kato K, Tohya Y, Fukushi S, Saijo M, Yoshikawa Y, Kurane I, Morikawa S, Akashi H	Ligation-mediated amplification for effective rapid determination of viral RNA sequences (RDV)	J. Clin. Virol.	43	56-59	2008
Yamao T, Eshita Y, Kihara Y, Satho T, Kuroda M, Sekizuka T, Nishimura M, Sakai K, Watanabe S, Akashi H, Rongsriyam Y, Komalamisra N, Srisawat R, Miyata T, Sakata A, Hosokawa M, Nakashima M, Kashige N, Miake F, Fukushi S, Nakauchi M, Saijo M, Kurane I, Morikawa S, Mizutani T	Novel virus discovery from field-collected mosquito larvae using an improved system for rapid determination of viral RNA sequences (RDV ver4.0).	Arch Virol.	154	153-158	2009

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A. Furuta, Y. Konodo, T. Saito, M. Tomita, K. Oka, Y. Kishimoto, Y. Tani & H. Shibata.	Transfusions of red blood cells from an occult hepatitis B virus carrier without apparent sighns of transfusion-transmitted hepatitis B infection.	Transfusion Medicine	18	379-381	2008
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Hayashi, K. Yasui, N. Matsuyama, R. A. Furuta, Y. Hori, S. Tanaka, F. Hirayama, Y. Tani, H. Shibata & M. Inoue.	Establishment of a novel method for detecting Nak^a antibodies by using a panel cell line.	Transfusion	49	390-392	2008

紀野修一、友田豊、遠藤玲美、他	輸血前血清を凍結保管していたことでB 型肝炎ウイルス再活性化の経過を調査しえた1 例.	日本輸血・細胞治療学会誌	53	553-557	2007
紀野修一	当院における輸血前・輸血後感染症検査実施のための取り組みと日本の現状	医学のあゆみ	225	610-611	2008
友田豊、紀野修一、森清香、花田大輔、武田悟、伊藤喜久	輸血事故防止のための院内体制整備	臨床検査	52	169-175	2008

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Novel reovirus isolation from an Ostrich (*Struthio camelus*) in Japan

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ABSTRACT

An orthoreovirus was isolated from an Ostrich (*Struthio camelus*) and rapidly identified as orthoreovirus by the rapid determination of viral RNA sequences (RDV) system and electron microscopy. Phylogenetic analysis of the sigma A protein indicated that the isolate belonged to avian species and was closely related to chicken orthoreovirus strain 138. The results of the present study indicated that an ostrich orthoreovirus is slight different from other chicken orthoreoviruses and provided evidence of diversity among avian orthoreoviruses. To our knowledge, this is the first genetic report of an orthoreovirus isolated from an ostrich.

1. Introduction

Commercial ostrich farms were introduced in Japan in 1988, and the number of ostriches increased rapidly over the next 15 years, exceeding 9000 birds by 2002. As ostriches are kept mainly under free-range conditions, they can easily come into contact with wild birds and animals that may be infected with pathogens, including bacteria, viruses and parasites. In our previous serological surveillance of Newcastle disease virus (NDV) in Japanese ostriches, 12.2% of 181 slaughter-age ostriches were shown to contain NDV-specific neutralizing antibodies without any vaccination history (Sakai et al., 2006). Also, NDV was also isolated from wild birds that may have come into contact with these ostriches (Sakai et al., 2007b). These observations indicated that NDV has infiltrated into ostrich farms in Japan. However, little information is available regarding infectious diseases other than Newcastle disease and avian influenza in ostriches. As the first step to identify unidentified viruses in ostriches, we attempted to isolate viruses and to accumulate information regarding viral nucleic acid sequences using a rapid determination of viral RNA sequences (RDV) system that has been used successfully for identification of RNA viruses (Mizutani et al., 2007; Sakai et al., 2007a; Kihara et al., 2007; Maeda et al., 2008; Watanabe et al., 2008).

2. Materials and methods

2.1. Virus isolation and virological characterization

Fresh intestinal content samples of slaughter-age ostriches (12 months old, apparently healthy) were obtained from ostrich breeder farm in Yamagata prefecture in Japan and put into PBS containing antibiotics (penicillin 10,000 units/ml, streptomycin 10,000 μg/ml, gentamycin 5000 μg/ml, amphotericin B 50 μg/ml) to give a 20% suspension. The suspension was centrifuged at 12,000 × *g* for 5 min. Aliquots of 200 μl of the supernatant were inoculated into primary chicken kidney cells (CKC) according to the methods described previously (Kawamura et al., 1965).

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Table 1
Primers used for the rapid determination of viral RNA sequences system

Sequence code	HaeIII primer sequence (5′–3′)	AluI primer sequence (5′–3′)
Forward primer		
H1-1	AATTCGGCGGCCGCGGATCCCCGGGG	AATTCGGCGGCCGCGGATCCCTGGGG
Reverse primer		
H9-1	AATTCGGCGGCCGCGGATCCCCAGGG	AATTCGGCGGCCGCGGATCCCTAGGG
H9-2	AATTCGGCGGCCGCGGATCCCCAGGC	AATTCGGCGGCCGCGGATCCCTAGGC
H9-3	AATTCGGCGGCCGCGGATCCCCAGGA	AATTCGGCGGCCGCGGATCCCTAGGA
H9-4	AATTCGGCGGCCGCGGATCCCCAGGT	AATTCGGCGGCCGCGGATCCCTAGGT
H9-5	AATTCGGCGGCCGCGGATCCCCAGCG	AATTCGGCGGCCGCGGATCCCTAGCG
H9-6	AATTCGGCGGCCGCGGATCCCCAGCC	AATTCGGCGGCCGCGGATCCCTAGCC
H9-7	AATTCGGCGGCCGCGGATCCCCAGCA	AATTCGGCGGCCGCGGATCCCTAGCA
H9-8	AATTCGGCGGCCGCGGATCCCCAGCT	AATTCGGCGGCCGCGGATCCCTAGCT
H9-9	AATTCGGCGGCCGCGGATCCCCAGAG	AATTCGGCGGCCGCGGATCCCTAGAG
H9-10	AATTCGGCGGCCGCGGATCCCCAGAC	AATTCGGCGGCCGCGGATCCCTAGAC
H9-11	AATTCGGCGGCCGCGGATCCCCAGAA	AATTCGGCGGCCGCGGATCCCTAGAA
H9-12	AATTCGGCGGCCGCGGATCCCCAGAT	AATTCGGCGGCCGCGGATCCCTAGAT
H9-13	AATTCGGCGGCCGCGGATCCCCAGTG	AATTCGGCGGCCGCGGATCCCTAGTG
H9-14	AATTCGGCGGCCGCGGATCCCCAGTC	AATTCGGCGGCCGCGGATCCCTAGTC
H9-15	AATTCGGCGGCCGCGGATCCCCAGTA	AATTCGGCGGCCGCGGATCCCTAGTA
H9-16	AATTCGGCGGCCGCGGATCCCCAGTT	AATTCGGCGGCCGCGGATCCCTAGTT

2.2. Rapid determination of viral RNA sequences (RDV) system

To identify the isolated virus, the rapid determination of viral RNA sequences (RDV) system version 2.1 (Sakai et al., 2007a and unpublished data) was applied. Briefly, culture supernatant was treated with DNase I and RNase A, following RNA extraction by ISOGEN-LS (Nippon Gene, Tokyo, Japan). Aliquots of 10 μl of RNA solution were amplified using a whole transcriptome amplification

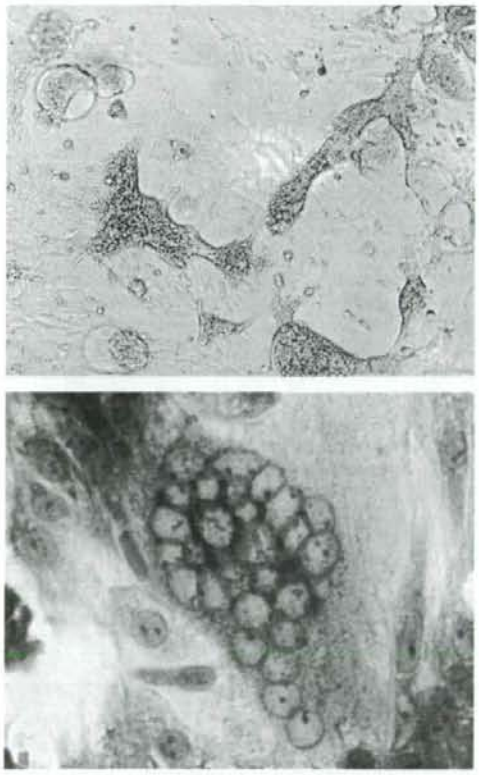

Fig. 1. Syncytia formation produced by infection of chicken kidney cells with Ost1 isolated from the ostrich.

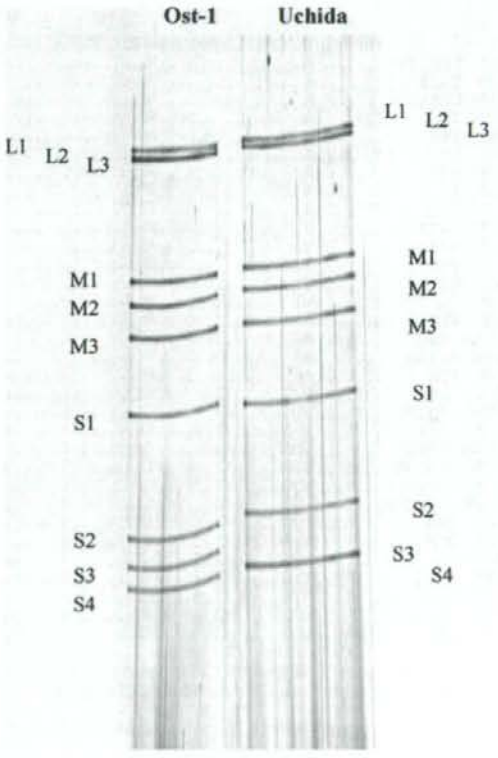

Fig. 2. Electrophoretic patterns of the genomic RNA of the Ost1 and avian orthoreovirus strain Uchida.

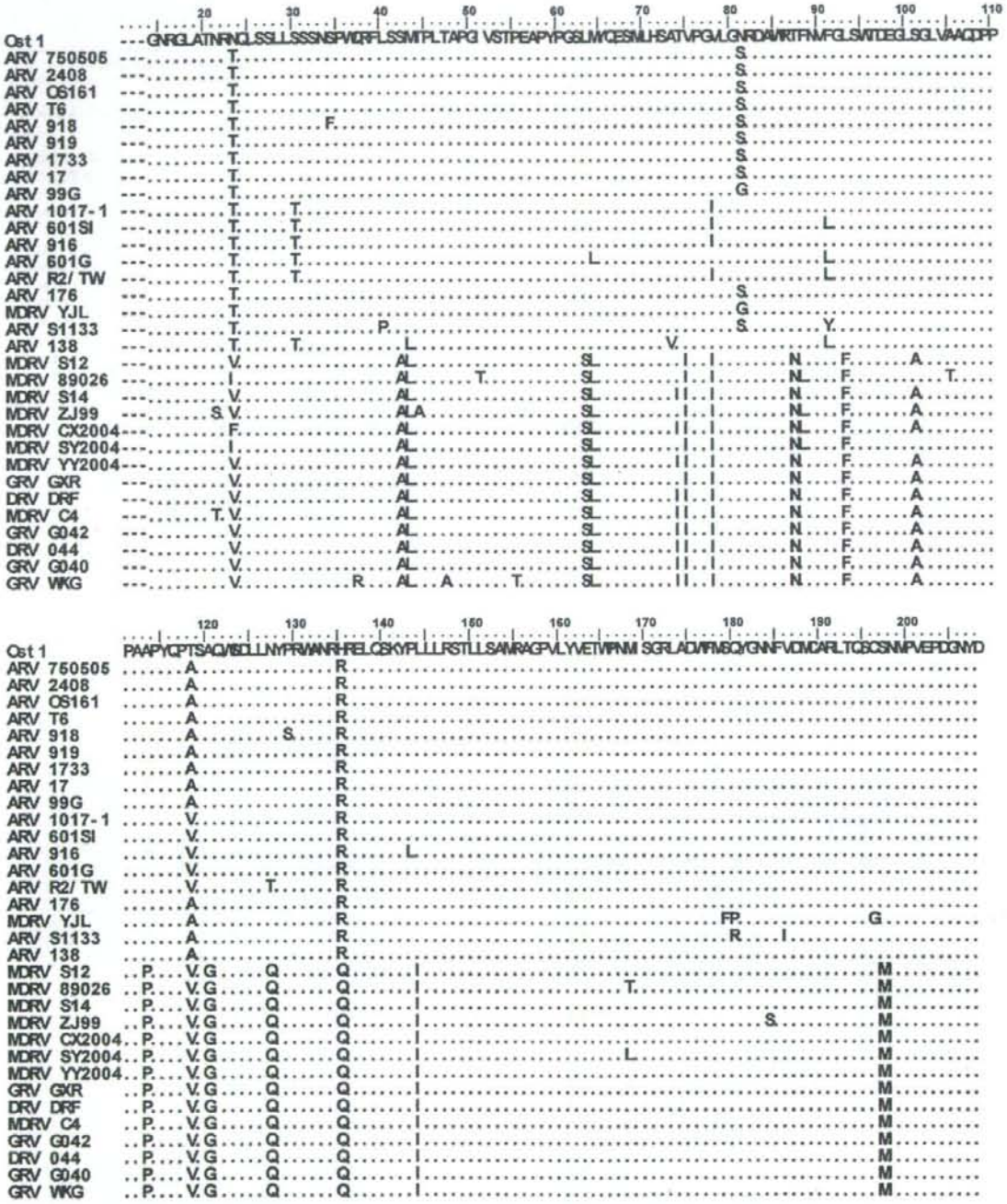

Fig. 3. Multiple alignments of the predicted σA amino acid sequences of avian, duck, and ostrich orthoreovirus strains (amino acids 14–208). ARV: avian (chicken) reovirus, MDRV: muscovy duck reovirus, GRV: goose reovirus, DRV: duck reovirus.

system (WTA; Sigma–Aldrich, Saint Louis, MO, USA) as first cDNA library. As described in our previous report (Sakai et al., 2007a), we used AmpliTaq Gold LD (Applied Biosystems, Foster City, CA, USA) to obtain a high yield of the WTA products. After the amplicon was digested with HaeIII or AluI, EcoRI-NotI-BamHI adaptor (Takara Bio Inc., Shiga, Japan) was ligated to the DNA fragments, and the second cDNA library was then amplified using AmpliTaq

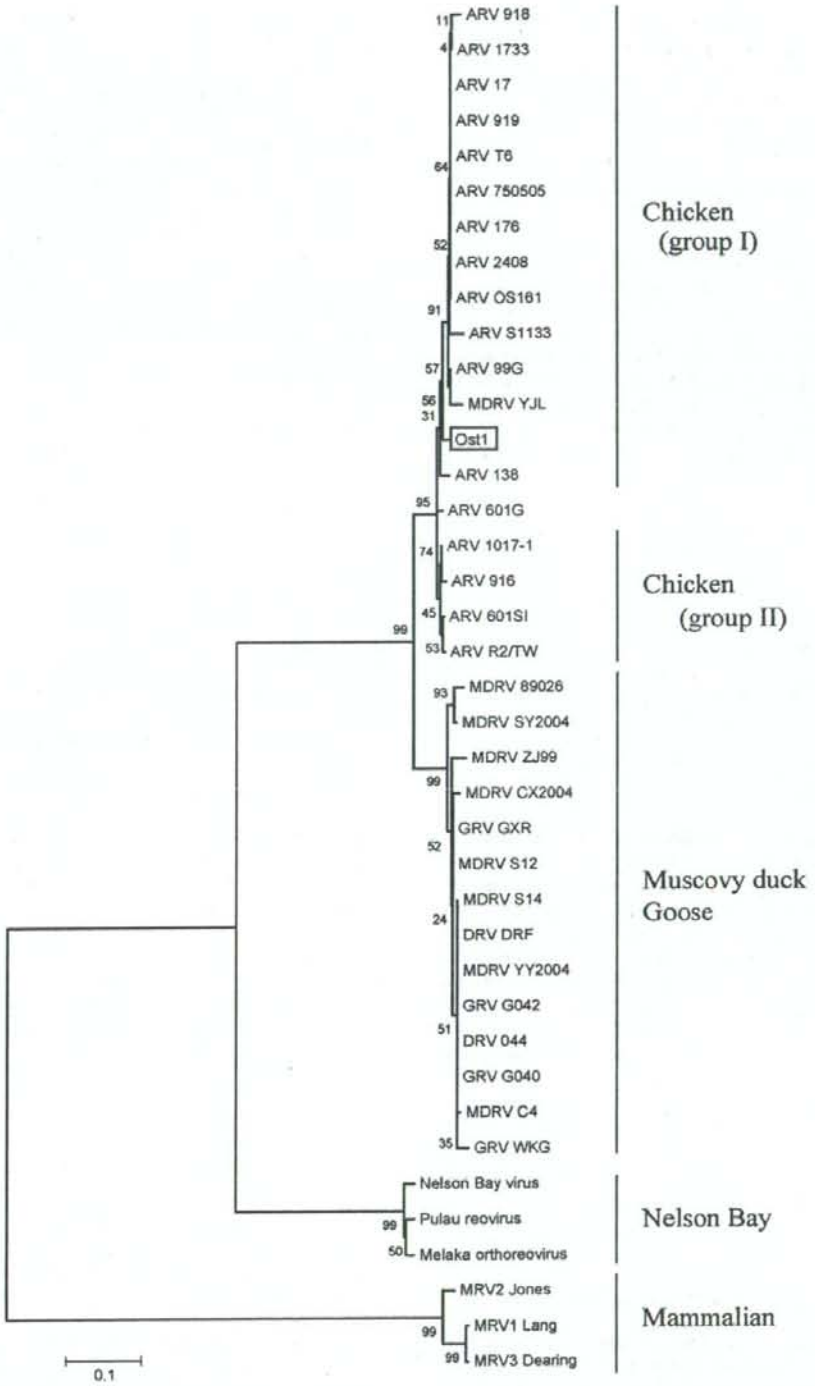

Fig. 4. Phylogenetic analysis of the orthoreovirus sigma A protein. Evolutionary distances were computed using the Poisson correction method and are in given in units of the number of amino acid substitutions per site using MEGA4. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

Gold PCR Master Mix (Applied Biosystems, Foster City, CA, USA) using the specially designed primer sets shown in Table 1. In this study, we used H1-1 primer as forward primer and H9-1 to -16 primers as reverse primers as described in Table 1. Direct sequencing was performed using the forward primers.

2.3. Preparation and analyses of viral double-stranded (ds) RNAs

Viral dsRNAs were purified as described (Moss et al., 1988). Briefly, viruses were inoculated into chicken kidney cell cultures at m.o.i. of 0.1 PFU per cell. When CPE appeared, the cells were harvested, resuspended in 10 mM Tris–HCl (pH 7.6), 1 mM EDTA (TE) buffer, and sonicated three times for 20 s each time. After clarification, the pellet was washed twice in TE buffer. The pooled supernatants were adjusted to 1% SDS and 0.4 M NaCl, extracted with phenol/chloroform, and precipitated with ethanol. The pellet was dissolved in a small amount of 1 mM EDTA (pH 5.0), and added an equal volume of 4 M LiCl. After 8 h at 4 °C, the single-stranded (ss) RNA was pelleted at 1000 × g for 30 min. An equal volume of 8 M LiCl was added to the supernatant and incubated for 8 h at 4 °C. The dsRNA was pelleted, dissolved in TE buffer, and precipitated with ethanol. The resultant dsRNA samples were mixed with loading buffer containing 0.05% (w/v) bromophenol blue and 10% (v/v) glycerin, and subjected to SDS-PAGE in 10% polyacrylamide gels. After electrophoresis the gels were silver stained using a Silver Stain Kit (ATTO, Tokyo, Japan).

2.4. Reverse transcription (RT)-PCR and sequencing, phylogenetic analysis

To confirm that the RNA specimen contained genomic RNA of orthoreovirus, we carried out RT-PCR using SuperScript III (Invitrogen, San Diego, CA, USA) for RT and Expand High Fidelity PCR system (Roche, Mannheim, Germany) for PCR using primers for avian orthoreovirus-consensus sequences within the segment S2 gene encoding the sigma A protein (Zhang et al., 2006). We obtained a single band at approximately 550 bp (data not shown) and determined the nucleic acid sequence by direct sequencing using the forward and reverse primers. The determined nucleotide sequences were analyzed with SEQUENCHER for Windows V4.7 Gene Codes Corporation, Ann Arbor, MI, USA. Phylogenetic analyses were carried out with the software program MEGA3 (Kumar et al., 2004). The sequence data of the segment S2 reported in this study were obtained from GenBank.

3. Results

3.1. Determination of viral RNA sequences by the RDV method

On the second passage of the primary cells, a cytopathic effect (CPE) was observed. In Fig. 1, two different major changes cultures were observed in the infected CKC: (i) formation of large thick appearing syncytia and (ii) condensation of cytoplasmic organelles including polynuclei that are encircled in a tiny ring-like structure. The virus, tentatively named as ostrich virus 1 (Ost1). We performed RDV method using purified RNA derived from the isolated virus. Seven cDNA fragments were homologous to the genes of avian orthoreoviruses isolated from chicken. Four cDNA fragments (DDBJ/EMBL/GenBank accession nos. AB435364, AB435365, AB435366 and AB435367) were homologous to the gene encoding lambda A protein of chicken orthoreovirus. Two cDNA fragments (DDBJ/EMBL/GenBank accession nos. AB435369 and AB435369) were homologous to the gene encoding lambda C protein. One cDNA fragment (DDBJ/EMBL/GenBank accession no. AB435370) was to homologous to the gene encoding sigma A protein. This result indicated that the isolated virus belongs to the family *Reoviridae*.

3.2. Electrophoretic analysis

At the same time, we performed electron microscopic analysis and PAGE analysis of viral RNA. Electron microscopy of purified Ost1 indicated that Ost1 accumulated in the cytoplasm and the size of capsids was 60–70 nm (data not shown). Electrophoretic patterns of the genomic RNA of Ost1 showed similar genomic profiles to chicken orthoreovirus strain Uchida, obtained from Kawamura et al. (1965) (Fig. 2). Although segments L1, L2, and L3 of both strains, and segment S3 and S4 of strain Uchida were not fully separated, only segment S3 showed differences in electrophoretic mobility. These features indicated that Ost1 has characters of an orthoreovirus.

3.3. RT-PCR, sequencing, and phylogenetic analysis

Sequence analysis of segment S2 (DDBJ/EMBL/GenBank accession no. AB435371) showed that Ost1 had the highest degree of similarity to chicken orthoreovirus strain 176 (ARV 176, DDBJ/EMBL/GenBank accession no. AF059716) and chicken orthoreovirus strain 17 (ARV 17, DDBJ/EMBL/GenBank accession no. AY962258) with 93.7% nucleotide identity. Insertions and deletions were not found the amino acid sequences in Ost1 and any avian orthoreovirus isolates (Fig. 3). In addition, a phylogenic tree based on amino acid sequences indicated that this Ost1 belongs to the family *Reoviridae*, genus *Orthoreovirus*, and Ost1 was closely related to chicken orthoreovirus strain 138 (ARV 138, DDBJ/EMBL/GenBank accession no. AF059717) isolated from the hock joint of an infected chicken in New Brunswick, USA (Fig. 4).

4. Discussion

In Zimbabwe, 28 of 149 apparently healthy ostriches (19%) were shown to contain antibodies to avian orthoreovirus using a modified commercial enzyme-linked immunosorbent assay (Cadman et al., 1994). Also, Jensen et al. (1992) and Allwright (1996) referred to signs, gross pathology and diagnosis of disease and properties of the reovirus. It appears that reoviruses were present already in Zimbabwe, Southern Africa and TX, USA. However, to our knowledge, there have been no previous reports of the isolation of orthoreoviruses from ostriches, and no sequences have been deposited in the databases. Here, we

isolated a novel ostrich orthoreovirus, Ost1, which was slight different from other avian orthoreoviruses and could be classified as an avian species (chicken group I) in the genus *Orthoreovirus*. Generally, avian orthoreoviruses cause high morbidity in young birds, in contrast to adult birds. In fact, the ostrich from which Ost1 reovirus was isolated was apparently healthy. As many avian orthoreoviruses are poultry pathogens of economic importance. Avian reovirus is important cause of diseases in poultry. In particular, reovirus-induced arthritis, chronic respiratory diseases, and malabsorption syndrome (Fahey and Crawley, 1954; Hieronymus et al., 1983) provoke considerable economic losses. Further studies are needed to determine the pathogenicity of Ost1 for ostrich chicks and other avian hosts. In addition, studies of sequence diversity of ostrich orthoreovirus and other viruses should increase our understanding of the disease of ostriches and contribute to the development of vaccines against these viral pathogens.

5. Conclusion

An orthoreovirus was isolated from an Ostrich (*Struthio camelus*) and confirmed using RDV method and electron microscopy. Phylogenetic analysis indicated that the isolate belonged to avian species and was closely related to chicken orthoreovirus strain 138.

Acknowledgments

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Short Communication

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

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

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

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

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

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

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

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

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

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

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

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

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

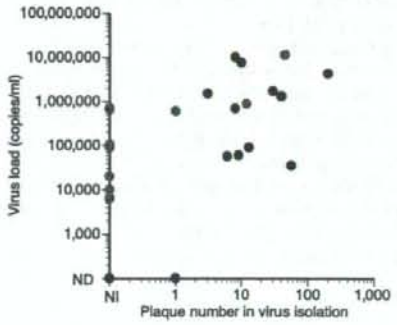

Fig. 1. Relationship between the virus load determined by the LC-qPCR and the plaque number in the virus isolation among PBC specimens. The specimens, which showed positive reactions in either virus-isolation or LC-qPCR, collected between day 0-10 and day 11-21 are shown in black circle and gray circle,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

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

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

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

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

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

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

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Direct Metagenomic Detection of Viral Pathogens in Nasal and Fecal Specimens Using an Unbiased High-Throughput Sequencing Approach

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Abstract

With the severe acute respiratory syndrome epidemic of 2003 and renewed attention on avian influenza viral pandemics, new surveillance systems are needed for the earlier detection of emerging infectious diseases. We applied a "next-generation" parallel sequencing platform for viral detection in nasopharyngeal and fecal samples collected during seasonal influenza virus (Flu) infections and norovirus outbreaks from 2005 to 2007 in Osaka, Japan. Random RT-PCR was performed to amplify RNA extracted from 0.1–0.25 ml of nasopharyngeal aspirates (N = 3) and fecal specimens (N = 5), and more than 10 μg of cDNA was synthesized. Unbiased high-throughput sequencing of these 8 samples yielded 15,298–32,335 (average 24,738) reads in a single 7.5 h run. In nasopharyngeal samples, although whole genome analysis was not available because the majority (>90%) of reads were host genome–derived, 20–460 Flu-reads were detected, which was sufficient for subtype identification. In fecal samples, bacteria and host cells were removed by centrifugation, resulting in gain of 484–15,260 reads of norovirus sequence (78–98% of the whole genome was covered), except for one specimen that was under-detectable by RT-PCR. These results suggest that our unbiased high-throughput sequencing approach is useful for directly detecting pathogenic viruses without advance genetic information. Although its cost and technological availability make it unlikely that this system will very soon be the diagnostic standard worldwide, this system could be useful for the earlier discovery of novel emerging viruses and bioterrorism, which are difficult to detect with conventional procedures.

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Introduction

Acute respiratory infections and diarrhea are the leading causes of childhood morbidity and mortality worldwide, each resulting in an estimated nearly 2 million deaths annually [1,2]. The diagnosis of respiratory and gastric/digestive infections is complex, due to the wide range of potential pathogens that can present with the same clinical symptoms [3]. In addition to the many known causes of these infections, it has been suggested that unrecognized infectious agents, including viruses, remain to be discovered [4]. It is estimated that, on average, up to 40% of diarrhea cases are of unknown etiology [4] and that the majority (69%) of upper respiratory infections are caused by viruses, including undiscovered ones [1].

Nucleic acid amplification tests (NATs) are increasingly being used for the diagnosis of viral infections. The most familiar formats use DNA or RNA target amplification methods, such as reverse transcription (RT) PCR, and have sensitivities that are greater than culture- or antigen-based procedures [3]. Loop-mediated isothermal amplification is more convenient and sensitive than PCR in amplifying DNA targets, and can be combined successfully with an RT step for RNA respiratory viruses. However, the wide variety of potential pathogens that elicit similar clinical symptoms and diseases makes the application of individual DNA- or RNA-based diagnostic assays both complex and expensive. Even multiplex PCRs are limited to 20–30 candidate pathogens, and may be confounded if viral evolution

results in mutations at the primer binding sites [2]. DNA microarrays offer unprecedented opportunities for multiplexing; however, they are not widely implemented in clinical microbiology laboratories because of problems with sensitivity, throughput, and validation [2]. In addition, these microarrays are unavailable for unknown and/or unexpected microbes, as they require genetic information for each tested pathogen.

Newly-developed "next-generation" sequencing technologies, such as 454 (Roche), Solexa (Illumina), and SOLiD (ABI), allow researchers, in an unbiased manner, to obtain millions of sequences in a single round of operation [5]. Among these sequencing technologies, 454 currently offers by far the longest read length, ~250 bp on the Genome Sequencer (GS) FLX platform [6]. Sequencing error levels are low (<1%) and arise primarily from homopolymer runs [7], but tend to be resolved in cases where there is sufficient coverage depth to allow the assembly of overlapping reads [8]. Many studies have used 454 pyrosequencing for the analysis of PCR amplicons, bacterial artificial chromosomes, genomic, mitochondrial, plastid DNA, and expression profiling [9,10,11,12,13,14]. 454 is also a powerful tool for pathogen discovery [15], and was used with the GS platform to identify a new arenavirus transmitted through solid-organ transplantation [16] and a new polyomavirus in samples of Merkel cell skin carcinoma [17]. The 454 sequencing technique was also used to implicate Israeli acute paralysis virus as a significant marker for colony collapse disorder in honey bees [18]. Another group reported the whole genome analysis of Gallid herpesvirus, and showed that >99.0% coverage was obtained by assembling the raw sequence data to an overall average coverage depth of 13 [19].

We previously demonstrated the direct detection of a bacterial pathogen from a patient sample using 454 high-throughput DNA sequencing [20]. Here we report the design and diagnostic validation of an unbiased high-throughput sequencing method for the direct diagnosis of viral infections in clinical specimens. Patient samples were obtained during seasonal influenza virus (Flu) infections and norovirus outbreaks from 2005 to 2007 in Osaka, Japan. cDNAs, as templates for the GS FLX platform, were prepared by random RT-PCR using RNAs extracted from clinical samples. High-throughput sequencing yielded 15,298–32,335 sequences, of which 7–15,260 represented the targeted viral sequences. Furthermore, sequences of two recently identified human viruses, WU polyomavirus (WUV) and human coronavirus (HCoV) HKU1, were detected from the nasal and fecal samples, respectively.

Results

Random RT-PCR Amplification by the Transplex WTA Kit

Total RNA isolated from either nasopharyngeal aspirates or fecal samples (0.1–0.2 ml) was under-measurable with an ND-1000 spectrophotometer (NanoDrop Technologies). Therefore, we performed quasi-random RT-PCR amplification using a whole transcriptome amplification (WTA) kit, according to the manufacturer's protocol with modifications, i.e. 70 cycles of PCR [21]. After random RT-PCR amplification, 10–13 μg of cDNA were obtained from the nasal and fecal samples (Figure 1A).

Semi-quantitative PCR was performed using 10-fold serial dilutions of the amplified cDNA as templates. Flu-specific PCR detected positive signals in all three nasopharyngeal aspirates (Figure 1B), and norovirus-specific primer sets detected the norovirus genome in four fecal samples, excluding #N1 sample (Figure 1C). The endpoint of detection in sample #N3 without 70 cycles of PCR amplification was E+03 (Figure 1C, left panel), whereas that with PCR amplification was E+06 (Figure 1C, right panel), suggesting that the viral cDNA was amplified almost 1,000 times by the 70 cycles of PCR. Together with the results that total RNA/cDNA were also amplified from several nanograms (data not shown) to ~10 μg by random RT-PCR, these results indicate that viral genomes can be amplified similar to other DNAs with the WTA kit. Because almost all of the amplified cDNA were within the 200–1,000 bp range (Figure 1A), the PCR products were directly used as templates for emulsion PCR in the GS FLX pyrosequencing.

Pyrosequencing Using the GS FLX Platform

The GS FLX system produces several million bases in one 7.5 h run [20]. The PicoTiterPlate device, physical divided into 16 regions, was used in this study, with 8 samples being loaded into 2 regions each. A single run yielded 15,298–32,335 (average 24,738) reads. Data analysis was basically performed according to the protocol we previously reported [20]. However, since the template cDNA for the high-throughput sequencing was prepared with random RT-PCR, an extra step was added to remove tag sequences in order to use the sequence data for BLASTN search analyses. Figure 2 shows the fraction of organisms (from which the sequences in the database were derived) that showed the best hits for the query sequences (E-value$<10^{-5}$). To identify viral species including subtype, higher matches (E-value$<10^{-40}$) for the sequence reads were selected (Table 1).

Nasal Samples

The Flu sequence was detected from all three samples in 21,858–30,958 (average 25,978) reads, as shown in Table 2. A partial genome was covered in these samples, and the cover rate ranged 8.1–58.3% (Table 2). One major reason for the partial coverage might be the large amount of host-derived sequences (90.0–94.6%; Table 2 and Figure 2), which were due to our direct RNA isolation from nasopharyngeal aspirates without the elimination of host or bacterial cells. However, 20–460 reads were Flu-derived, which was sufficient for subtype identification (H1N1 in sample #F2 and H3N2 in samples #F1 and #F3) from these sequences (Table S1).

In addition to the Flu sequence, a WUV-derived sequence was detected in one specimen (#F3) (Figure 3A). Because the detected sequences were located in a single gene (VP1), the presence of a second gene (VP2) was confirmed with PCR (Figure 3B). WUV and another novel human polyomavirus KI were cloned from respiratory tract specimens in 2007 [20,22,23,24]. Although their etiological role in childhood respiratory disease has been proposed [23,24], inconsistent epidemiological results have been reported [25]. In this study, the WUV-positive patient was a kindergarten student who was co-infected with Flu, consistent with the report by Norje et al. [25]. Partial sequences of the human endogenous retrovirus HCML-ARV were detected in sample #F1 (Table 1), although the pathogenesis of this virus is unknown.

The number of bacterial sequences read was 572 (#F1), 230 (#F2), and 272 (#F3), and the relative ratio to the total number of reads was 1.9%, 1.2%, and 1.2%, respectively (Table 2). The most abundantly detected bacterial sequences were *Streptococcus pneumoniae*, *Moraxella bovis*, *Moraxella bovoculi*, *Haemophilus influenzae*, and *Escherichia coli* (data not shown), which were present as major bacteria in the respiratory tract of children.

Fecal Samples

To remove bacteria and human cells present in the feces, 15,000 rpm centrifugation was performed and the supernatants were used for RNA isolation. The norovirus sequence was detected from all five samples in 15,298–32,335 (average 23,994) reads, as summarized in Table 3. In contrast with influenza virus, almost the whole genome was covered in #N2 (7,302 reads) and

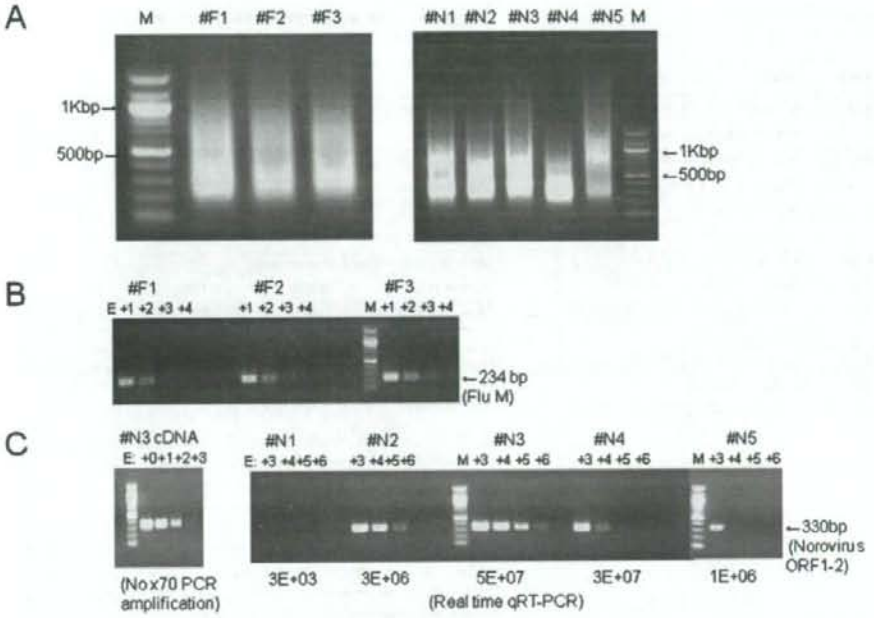

Figure 1. Random RT-PCR amplification of cDNA from clinical specimens and quantitative RT-PCR with virus-specific primers. The samples were nasopharyngeal aspirates and stools (n = 3 and 5, respectively) isolated during 2005–2007 in Osaka, Japan. Influenza A virus and norovirus were detected in the nasopharyngeal aspirates and stool samples, respectively, with other diagnostic methods. **(A)** RNA extracted from clinical specimens was reverse-transcribed and random-PCR amplified to prepare template DNA for pyrosequencing. One microgram of amplified PCR products in each sample was loaded onto a 1% agarose gel. M indicates 100-bp DNA ladder (NEB). **(B)** Flu-specific semi-quantitative PCR was performed with 10-fold serial dilutions of the random-PCR products. **(C)** Norovirus (GII)-specific semi-quantitative PCR was performed with 10-fold serial dilutions of the random-PCR products. Quantitative real-time RT-PCR using a norovirus-specific primer set was also performed, and the estimated copy numbers of norovirus in samples #N1–#N5 are shown in the panel on the right. As a control, cDNA from sample #N3 without random PCR amplification was used (left panel).
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#N3 (15,260 reads) samples, with average cover depths of 141.5 and 258.7, respectively (Table 3). More than 75% of the genome was covered in #N4 (484 reads) and #N5 (611 reads) samples (Table 3). A BLAST search of each sequence strongly indicated that these four patients were infected with a similar genotype, GII.4 (Table S2), consistent with previous diagnostic results [26]. In contrast, only 7 reads were detected in sample #N1 (Table 3), which was under-detectable with single round of PCR (Figure 1C), suggesting that the diagnostic method using high-throughput pyrosequencing is more sensitive than conventional PCR analysis.

Figure 2. Pyrosequencing using the GS FLX system. Amplified cDNA was used as a template for GS FLX analysis. A 70×75 PicoTiterPlate device (gasket for 16 regions) was divided into 2 regions each for 8 samples. Obtained data were then subjected to a data analysis pipeline, as described in the Methods section. Comparison of the organisms from which the best matches for the sequences was shown.
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One-step real time RT-PCR (qRT-PCR) was also performed on the extracted RNA. The estimated copy number of norovirus in each fecal sample is shown in Figure 1C. The copy number of norovirus in sample #N1 with qRT-PCR was 3E+03, whereas those of other samples ranged from 1E+06 to 5E+07. The relative copy numbers of norovirus in samples #N1 to #N3, which were isolated in 2006 (October to December), were almost consistent with the semi-quantitative PCR results, although the sensitivity of the semi-quantitative PCR was 10-fold lower than that of qRT-PCR (Figure 1C). By contrast, the real-time RT-PCR and semi-quantitative PCR were ~1,000-fold different in samples #N4 and #N5 (Figure 1C), which were isolated in May 2005 and January 2006, respectively. In these samples, RNA isolation for 454 pyrosequencing was performed in February 2007, whereas RNA for qRT-PCR was isolated just after sample collection. Thus, the above inconsistencies might be due to the storage periods of the samples (at 4°C).

HCoV-HKU1, which was recently identified as the fifth human coronavirus [27], was detected in one specimen (#N4). A total of 14 reads from 18,014 reads matched to the HCoV-HKU1 virus, with four regions being detected (Table 4). Epidemiological studies have reported that HCoV-HKU1 was found in the nasopharyngeal aspirates of 10/418 (2.4%) studied patients with community-

Table 1. Summary of detected viruses.

Sample	Age	Read	Virus
#F1	3	460	Influenza A virus (H3N2)
		3	Human endogenous retrovirus HCML-ARV
#F2	7	20	Influenza A virus (H1N1)
#F3	5	107	Influenza A virus (H3N2)
		7	WU Polyomavirus
#N1	62[a]	7	Norovirus (GII/4)
#N2	82[b]	7,304	Norovirus (GII/4)
#N3	92[b]	15,272	Norovirus (GII/4)
		813	Kyuri green mottle mosaic virus
		7	Citrus tristeza virus
		3	Enterobacteria phage phiK
#N4	3[c]	484	Norovirus (GII/4)
		14	Human coronavirus HKU1
		3	Phage phiV10
		3	Human endogenous retrovirus K
#N5	44[d]	762	Pepper mild mottle virus
		611	Norovirus (GII/4)
		17	Crucifer tobamovirus
		2	Tobacco mosaic virus

[a]Hospitalized patient.
[b]Patients in welfare facilities.
[c]Kindergarten student.
[d]Putative food.
doi:10.1371/journal.pone.0004219.t001

Table 2. Summary of gene analysis in nasal samples.

Sample	#F1	#F2	#F3
Total reads	30,958 (100%)	25,119 (100%)	21,858 (100%)
Eukaryotes	27,849 (90.0%)	23,760 (94.6%)	20,296 (92.9%)
Bacteria	572 (1.85%)	230 (1.23%)	272 (1.24%)
RNA viruses	506 (1.63%)	21(0.10%)	121 (0.55%)
Others	2,031 (6.56%)	1,108 (7.63%)	1,169 (5.35%)
Influenza A virus	460 (1.49%)	20 (0.08%)	103 (0.49%)
Mapping to influenza A virus genome.			
Cover rate	58.30%	8.10%	25.60%
Avg. depth	3.67	0.23	0.65

The E value threshold was set to 1E+05 for taxonomy classification and 1E+40 for virus detection, respectively. Reference sequences used for mapping are CY026275-82, A/Texas/UR06-0566/2007(H3N2).
doi:10.1371/journal.pone.0004219.t002

acquired pneumonia [28], and that HCoV-HKU1 could be detected in respiratory and stool samples from children and adults. Studies have also reported a 9-month-old patient who was co-infected with HCoV-HKU1 and influenza C virus [29]. We showed here that a 5-year-old child was co-infected with HCoV-HKU1 and norovirus, although the relationship between these two viruses and/or the relationship between pathogenesis (enteric tract illness) and co-infection of these two viruses remains unknown. Other human coronaviruses (OC-43, 229E, and NL63) were not detected from these fecal samples or from the nasal samples with RT-PCR (data not shown). Human endogenous retrovirus K (HERV-K)-derived sequences was also detected in patient #N4, who was 3 years old (Table 1). HERV-K is the name given to an approximately 30-million-year-old family of endogenous retroviruses present at >50 copies per haploid human genome [30].

In addition to these human viruses, several plant virus–derived sequences were also detected in the fecal samples (Table 1). In particular, pepper mild mottle virus (PMMV) was found in two specimens (#N1 and #N5) and was confirmed with RT-PCR (data not shown). The total number of PMMV reads outnumbered the norovirus reads in sample #N5 (Table 1). In addition, Kyuri green mottle mosaic virus (KGMMV) was also abundantly detected in sample #N3 (813 KGMMV-specific reads in a total 25,500 reads). KGMMV was also detected after ultracentrifugation (data not shown), suggesting that KGMMV viral particles were present in the human gut. Previously, PMMV was detected at 10^9 virions per gram of dry weight fecal matter, and was detected in 12 (66.7%) of the 18 fecal samples collected from healthy humans [31]. This previous publication also showed that fecal PMMV was infectious to host plants [31]. Therefore, these plant-derived viruses may retain their infectivity in the feces and may even be present in diarrhea. Almost all of the detected viruses, except for the citrus tristeza virus, a member of the *Closterovirus* group, belong to the *Tobamovirus* group (Table 1). It is currently unknown if there is an interaction between these plus-stranded RNA viruses and norovirus. The most abundantly detected PMMV was also found in healthy humans [31]. Although the previous paper [31] reported that there is a lack of evidence to show that active replication of PMMV occurs in human feces, further investigations regarding plant virus replication in the human gut (epithelial cells) seem necessary.

As shown in Table 3, 3,039–23,955 (11.9–83.1%) reads were estimated to be bacterial genes. Of those, more than half (54.7–69.9%) were rRNA-derived (Table 5) and a BLAST searching predicted the existence of commensal bacteria in the human intestine (data not shown).

Discussion

In this study, we demonstrated the potential of the 454 parallel sequencing platform to identify pathogenic viruses from clinical specimens. We chose random RT-PCR for template cDNA preparation, because of the low levels of isolated RNA from the specimens. Flu and norovirus were detected from all three nasopharyngeal aspirates and five stool specimens, respectively, consistent with other diagnostic methods, including RT-PCR. In addition to these viruses, possible pathogenic viruses, such as WUV and HCoV-HKU1, were also detected in the nasopharyngeal aspirates and fecal samples, respectively, suggesting that this system (Figure 4) is useful for novel virus identification as well as for viral genome analysis.

In the severe acute respiratory syndrome (SARS)-CoV outbreak in 2003, it was demonstrated that a combination of stool, pooled nasal, and throat swab specimens gave the highest yield for SARS-CoV detection by RT-PCR [32]. Thus, not only respiratory specimens but also gastric and digestive specimens are important for the diagnosis of emerging infectious viruses, including those with airborne transmission. In this study, we isolated whole RNA and detected viral genes from nasal and stool samples with the 454 high-throughput sequencing system. Flu sequences were present in 20–460 of the 21,858–30,958 reads in each nasopharyngeal aspirate (Table 1), and the cover rates ranged from 8.1–58.3% (Table 2), which was sufficient for subtype identification in all three specimens. Furthermore, the near-complete norovirus genome sequence was obtained in two fecal specimens (#N2 and #N3), and more than 75% of the genome was covered in the other two specimens (#N4 and #N5) (Table 3).

A

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Query: 28   gggccgcaactccaccagtggtgtcatttagtaataatagcacaattccactgttggat 87
            ||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Subjct:2357 gggcctgcaactccaccagtggtgtcatttagtaataatagcacaattccactgttggat 2416

Query: 88   gaaaatggcattggcattctttgcttgcaaggtagattgtacataacttgtgctgaccttt 147
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subjct:2417 gaaaatggcattggcattctttgcttgcaaggtagattgtacataacttgtgctgaccttt 2476

Query: 148  ttgggagttaacaaaaatagagtacatacagggctttccagatttttttaggctacacttt 207
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subjct:2477 ttgggagttaacaaaaatagagtacatacagggctttccagatttttttaggctacacttt 2536

Query: 208  agacaaagaaggcttagaaacccatatac 236
            ||||||||||||||||||||||||||||
Subjct:2537 agacaaagaaggcttagaaacccatatac 2565  (VP1 gene)
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B

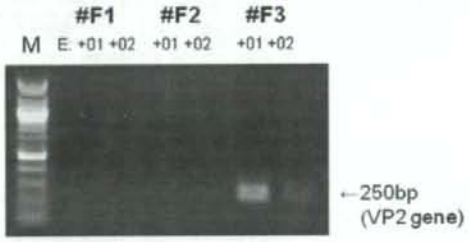

Figure 3. BLASTN (A) and PCR (B) analyses of WUV in Flu-positive nasopharyngeal aspirates. (**A**) Alignment of the WUV VP1 nucleotide sequence. The read obtained with the GS FLX sequencer (query) was compared with that of the WUV strain CLFF (subject; NCBI accession number: EU296475). (**B**) The WUV VP2 gene was detected by PCR using cDNA, which was amplified with random RT-PCR, as a template. The cDNA was diluted 10- and 100-fold and the PCR product was loaded on a 1% agarose gel. M indicates 100-bp DNA ladder.
doi:10.1371/journal.pone.0004219.g003

Recently, we subjected stool sample–extracted DNAs to 454 pyrosequencing, and found that nearly 20% of the reads had best hits that matched currently-reported bacterial DNA sequences [20]. These previous results, together with our findings here, indicate that two protocols, namely direct DNA extraction for bacteria and cell/bacterial removal by centrifugation followed by RNA/DNA extraction for virus, could be used to comprehensively identify pathogenic microbes in clinical samples.

Our preliminary experiments demonstrated that the detected number of viral sequences paralleled the virus copy number in blood samples (unpublished data), suggesting that this system is highly quantitative. Indeed, the copy number of norovirus in the #N1 to #N5 samples (Figure 1C), as measured by semi-quantitative PCR, was significantly correlated with the number of norovirus genome sequences as detected by high-throughput sequencing (Table 1). In the case of the nasal samples, the copy number of the influenza virus in sample #F2 was lower than those in samples #F1 and #F3, although the endpoints of the semi-quantitative PCR for these samples were comparable (Figure 1B). This inconsistency could be due to differences in the sensitivities of

Table 3. Summary of gene analysis in fecal samples.

Sample	#N1	#N2	#N3	#N4	#N5
Total reads	15,298 (100%)	32,335 (100%)	25,500 (100%)	18,014 (100%)	28,823 (100%)
Eukaryotes	400 (2.61%)	1,031 (22.7%)	147 (0.58%)	2,574 (14.3%)	948 (3.29%)
Bacteria	10,963 (71.7%)	14,423 (48.0%)	3,039 (11.9%)	9,180 (51.0%)	23,955 (83.1%)
RNA viruses	11 (0.07%)	8,742 (27.4%)	20,775 (81.5%)	546 (3.03%)	1,571 (5.45%)
Others	3,924 (25.7%)	8,139 (25.2%)	1,539 (6.04%)	5,714 (31.7%)	2,349 (8.15%)
Norovirus	7 (0.05%)	7,302(22.6%)	15,260 (59.8%)	484 (2.69%)	611 (2.12%)
Mapping to Norovirus genome					
Cover rate	2.10%	97.00%	98.00%	77.50%	84.50%
Avg. depth	0	141.5	258.7	9.3	12.5

Reference sequence used for mapping is AY587989, Norovirus Hu/NLV/Oxford/B2S16/2002/UK.
doi:10.1371/journal.pone.0004219.t003