Table 3. Clinical signs, virus replication, seroconversion, and direct contact transmission in ferrets inoculated with H7 influenza viruses

		No. of inoculated ferrets/total number					
		Clinical signs				No. of contact ferrets/total number	
Virus	Subtype	Weight loss, %*	Respiratory symptoms, day p.i.	Peak mean $\log_{10}$ nasal wash titer, day p.i.	Seroconversion (HI titer range)†	Virus detected in nasal wash	Seroconversion (HI titer range)†
NL/219	H7N7	3/3 (18.3)	3/3 (3)	6.3 (1,3)	1/1 (320)‡	0/3	0/3
NL/230	H7N7	3/3 (6.7)	1/3 (5)	6.5 (3,5)	3/3 (320–640)	2/3	2/3 (320, 640)
Can/504	H7N3	3/3 (17.2)	1/3 (5)	7.4 (3)	3/3 (320–640)	2/3	0/3
Tky/VA	H7N2	3/3 (6.6)	2/3 (3)	6.75 (1)	3/3 (320–640)	0/3	0/3
NY/107	H7N2	3/3 (6.3)	1/3 (5)	6.3 (1)	3/3 (640–1,280)	3/3	3/3 (1,280–2,560)
Ck/Conn	H7N2	2/3 (3.8)	1/3 (7)	7.1 (1)	3/3 (320–640)	1/3	1/3 (160)

*The percentage mean maximum weight loss is shown.
†HI assays were performed with homologous virus and horse RBCs.
‡Only one ferret survived and was tested.

model (13). In the current study, both NL/219 and NL/230 viruses replicated efficiently in the upper respiratory tract of inoculated ferrets, with peak mean nasal wash virus titers of 6.3 ± 0.2 and 6.5 ± 0.9 $\log_{10}$ EID_{50}/ml detected on day 3 p.i., respectively (Fig. 2 *A* and *B*). Two of three animals inoculated with NL/219 virus in each experiment were humanely killed 5–7 days p.i. because of severe weight loss or development of hind-limb paralysis. NL/219 virus did not transmit by either direct contact or respiratory droplets, because virus was not isolated from nasal washes of contact ferrets, and seroconversion of contact animals for hemagglutination inhibition (HI) antibody did not occur (Fig. 2*A*, Table 3, data not shown). Respiratory droplet transmission of NL/230 virus was not observed (data not shown); however, in the direct contact experiment, NL/230 virus was detected in the nasal washes of two of three contact ferrets, with peak NW virus titers $>10^{6.5}$ EID_{50}/ml by day 8 postcontact (p.c.) in these animals (Fig. 2*B*). Both NL/230 contact ferrets that had virus isolated from NW seroconverted by the end of the experiment (Table 3). The third NL/230 contact ferret did not have detectable virus in NW and did not seroconvert (Fig. 2*B*, Table 3). This pattern of NL/230 virus transmission by direct contact was confirmed in a duplicate experiment that resulted in seroconversion of only two of three ferrets. Taken together, these results indicate that, despite similar receptor-binding properties as measured by glycan array and resialyation assay, NL/230 virus exhibited an enhanced ability to transmit in the ferret model by direct contact compared with NL/219 virus.

Transmissibility of North American H7 Influenza Viruses in Ferrets. Next, we assessed the ability of the H7 viruses of the North American lineage to spread to naïve ferrets by either respiratory droplet or contact transmission. Tky/VA virus replicated efficiently in the upper respiratory tract of inoculated ferrets, with peak mean virus titers reaching 6.75 ± 0.5 $\log_{10}$ EID_{50}/ml on day 1 p.i. (Fig. 2*C*). However, Tky/VA virus did not transmit by direct contact, because virus was not isolated from nasal washes of contact ferrets, and seroconversion of contact ferrets was not detected (Fig. 2*C*, Table 3). Can/504, a HPAI H7N3 virus, was also found to replicate efficiently in the upper respiratory tract of inoculated ferrets, with peak mean nasal wash virus titers reaching 7.4 ± 0.3 $\log_{10}$ EID_{50}/ml on day 3 p.i. (Fig. 2*D*). Additionally, substantial weight loss was observed in ferrets inoculated with Can/504 virus (Table 3). Low levels of virus were detected in the nasal washes of two ferrets in direct contact with inoculated animals (1.98–2.25 $\log_{10}$ EID_{50}/ml); however, seroconversion of these contact ferrets did not occur (Table 3). These low virus titers are most likely due to the presence of residual virus on the noses of contact ferrets that was acquired from the environment or from the inoculated ferrets and therefore does not constitute efficient virus transmission, because sustained high titers of virus in the upper respiratory tract were not detected, and seroconversion did not occur. Respiratory droplet transmission of Tky/VA or Can/504 virus was not detected (data not shown).

As discussed above, two H7N2 viruses, NY/107 and Ck/Conn, exhibited enhanced α2–6 SA binding with decreased binding to α2–3 SA in the glycan microarray, with NY/107 showing the most significant decrease. Similar to all other H7 viruses tested, NY/107 and Ck/Conn viruses were detected at high titers in nasal washes of inoculated ferrets, with peak mean virus titers of 6.3 ± 0.5 $\log_{10}$ EID_{50}/ml and 7.1 ± 0.3 $\log_{10}$ EID_{50}/ml, respectively, on day 1 p.i. (Fig. 2 *E* and *F*). In contrast with Tky/VA or Can/504 viruses, NY/107 virus transmitted efficiently to three of three ferrets by direct contact, with peak virus titers in nasal washes from each contact ferret reaching $\geq 10^{5.25}$ EID_{50}/ml and seroconversion occurring in all contact animals (Fig. 2*E*, Table 3). Transmission by direct contact occurred by day 2 p.c. in one ferret and by day 6 p.c. in the remaining two contact animals (Fig. 2*E*). In comparison, Ck/Conn virus transmitted by direct contact in one of three contact ferrets, with peak NW virus titer reaching $10^{7.25}$ EID_{50}/ml on day 10 p.c. (Fig. 2*F*). Seroconversion of the remaining two contact ferrets did not occur, indicating that Ck/Conn virus, unlike NY/107 virus, did not transmit efficiently by direct contact (Table 3). Similar to other H7 viruses in this study, respiratory droplet transmission was not observed with either virus (data not shown). These findings demonstrate the ability of an H7 influenza virus isolated from a human, NY/107, to transmit efficiently by direct contact in the ferret model.

Discussion

Like other avian influenza viruses, those within the H7 subtype fall into two geographically distinct lineages, Eurasian and North American (35, 36). H7 viruses within these lineages have caused outbreaks and human infection in recent years and continue to pose a public health threat. To better assess the pandemic potential of H7 influenza viruses, we examined the receptor-binding preference and transmissibility of selected H7 viruses associated with disease in humans. We found that Eurasian lineage HPAI H7 influenza viruses tested in this study closely resemble recent HPAI H5N1 viruses with respect to their binding preference for α2–3 SA receptors. Conversely, we observed an increase in α2–6 binding among North American lineage H7 viruses isolated between 2002 and 2004. Several of these also showed reduced binding of α2–3 SA receptors characteristic of human influenza viruses. The most dramatic shift in receptor specificity was observed for a human H7 influenza virus that was also transmitted efficiently between animals by direct contact.

Previous studies have suggested that Eurasian lineage H7 influenza viruses share receptor-binding properties similar to H5 vi-

ruses, because analysis of the HA crystal structure derived from the H7 virus A/Tky/Italy/02 demonstrated specific binding to avian receptor and not human receptor analogues (37). Recent advances in glycan microarray technology allowed us to more closely analyze the fine differences in receptor specificity of viruses between both Eurasian and North American H7 viruses. Here, we used a whole-virus assay that allowed for examination of the binding properties of influenza viruses without the need for generation of recombinant HA. We were particularly interested in the North American H7 viruses, because some of these avian viruses appear to be adapted to the upper respiratory tract of chickens, which have been shown to express more α2–6 SA receptors compared with wild aquatic birds (38); *in vivo* studies have demonstrated that North American lineage LPAI H7N2 viruses replicate to high titer in the upper respiratory tract of chickens and turkeys compared with the gastrointestional tract (39–41).

All H7 viruses tested replicated to high titer in the upper respiratory tract in inoculated ferrets as shown (13); nevertheless, most isolates tested failed to transmit despite the high titers of virus shed by inoculated animals. Respiratory symptoms such as sneezing and nasal discharge were observed in some ferrets inoculated with each H7 virus (Table 3). However, the frequency and duration of these symptoms in this model more closely resembled those observed in ferrets inoculated with H5N1 viruses, rather than the more pronounced respiratory symptoms observed in ferrets infected with human H3N2 or H1N1 viruses (21, 23). With the exception of NL/219-inoculated ferrets, which exhibit substantial lethargy after infection (13), ferrets inoculated with H7 viruses in this study remained alert and playful for the duration of the experiment, suggesting that frequent interaction between inoculated and contact ferrets is not sufficient for virus transmission to occur. Additionally, the results of this study indicate that increased virus binding to α2–6 SA is not sufficient for transmission of avian influenza viruses to occur, supporting previous studies demonstrating the lack of transmission of an H5N1 virus with an increased α2–6-binding preference (21, 22). Recent studies have highlighted increased complexity of the structural topology among α2–3 and α2–6 SA and suggest that conformational features of the linkage contribute to virus binding and could play a role in virus transmissibility (42). This diversity of SA receptors could in part contribute to the enhanced ability of NL/230 virus to transmit in the ferret model by direct contact compared with NL/219 virus. Although the Eurasian lineage H7N7 viruses analyzed in this study displayed similar receptor-binding properties as measured by glycan array and hemagglutination assay, Munster *et al.* (43) found differential attachment of NL/219 virus and a virus closely related to NL/230 to tissues in the lower respiratory tract of humans. The enhanced transmissibility observed with NL/230 virus in this study compared with NL/219 virus would additionally suggest subtle differences in the receptor-binding properties between these H7N7 viruses that have yet to be identified.

Unlike most subtypes of influenza, infection with H7 influenza viruses frequently results in conjunctivitis in humans and not respiratory disease (6, 7, 9). Unlike the upper respiratory tract in humans, which contains a high distribution of α2–6 SA, corneal, conjunctival, and lacrimal duct epithelial cells of the human eye express predominantly α2–3 SA (27, 44, 45). Additionally, the sialylated secretions (mucins) of both surfaces differ in their SA content; mucins in the airway epithelium contain α2–3 SA, whereas ocular secreted mucins contain α2–6 SA (46–48). The high α2–3 SA content of the human ocular surface suggests that avian influenza viruses would be well suited to use this surface as a portal of entry. However, although human infection with H7 influenza viruses frequently results in conjunctivitis, documented cases of ocular disease after H5N1 infection are rare (7, 49, 50). The heterogeneity of SA-binding preference observed between H7 influenza virus lineages suggests that, similar to virus transmissibility, ocular tropism is a complex property that cannot be explained by SA receptor binding alone.

We identified a LPAI H7N2 virus, NY/107, which was associated with human respiratory infection and not ocular disease and was effectively transmitted in the ferret model by direct contact (10). Among all H7 viruses analyzed by glycan microarray, NY/107 displayed the most dramatic increase in α2–6 SA binding along with decreased α2–3 SA binding avidity. Strong α2–6 SA binding appears to be an essential component of conferring transmissibility in human influenza viruses, because H1N1 variant viruses exhibiting the classic avian α2–3-binding preference or dual α2–3- and α2–6-binding preference were unable to transmit efficiently (23). These results suggest that a decrease in α2–3 SA binding may also be needed in addition to α2–6 SA-binding avidity. However, despite similar α2–3 and α2–6 SA binding observed by glycan array with Ck/Conn virus, this virus was transmitted only by direct contact in one of three animals. Efficient contact transmission was also not observed with Tky/VA virus, despite this virus sharing 98.4% HA amino acid identity with NY/107 virus (40). Future studies will allow for a better understanding of the genetic determinants responsible for the heightened transmissibility observed with this virus. NY/107 virus, like all H7 viruses tested in this study, did not transmit by respiratory droplets in the ferret model. However, the efficient NY/107 virus transmission observed by direct contact in ferrets has not been observed with HPAI H5N1 viruses (21, 22) and may indicate the capacity of a NY/107-like virus to acquire properties that would confer efficient transmission by respiratory droplets; this underscores the importance of studying virus transmissibility by both routes.

LPAI H7N2 viruses have been acquiring additional basic amino acids at the HA cleavage site since 1994, resulting in a cleavage site that more closely resembles HPAI viruses (51). These viruses are also characterized by a deletion of 8 aa in the HA1 proximal to the receptor-binding site (31); further study will help elucidate whether this deletion contributes to the enhanced α2–6 SA binding observed among these viruses. The classic avian specificity for α2–3-linked SA observed with Rhea/NC could suggest a possible correlation between the acquisition of α2–6 SA binding and the introduction of LPAI H7N2 viruses into the live bird markets of the northeastern U.S. The finding of enhanced α2–6 SA binding of North American H7 viruses underscores the necessity for continued surveillance and study of these viruses as they continue to resemble viruses with pandemic potential.

Materials and Methods

Viruses. Virus stocks were grown in the allantoic cavity of 10-day-old embroynated hens' eggs as described (13). The 50% EID_{50} titer for each virus stock was calculated by the method of Reed and Muench (52), after serial titration in eggs. A/Texas/36/91 (Tx/91) stock was grown on Madin–Darby canine kidney cells containing DMEM, 0.025 M Hepes, 0.3% BSA (Gibco Invitrogen), and *N*-p-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Sigma–Aldrich). All experiments with HPAI viruses were conducted under biosafety level 3 containment, including enhancements required by the U.S. Department of Agriculture and the Select Agent Program (53).

Glycan Microarray Analysis. Analysis of the receptor specificity of influenza virus using glycan microarrays was done largely as described (33, 54). Custom arrays for influenza research were produced for the Centers for Disease Control and Prevention on National Health Service-activated glass slides (Schott Nexterion) by using a glycan library provided by the Consortium for Functional Glycomics [www.functionalglycomics.org; see supporting information (SI) Table S1 for a list of glycan structures]. Viruses were inactivated by treatment with β-propiolactone (0.001%) overnight at 4°C with virus inactivation confirmed by two rounds of passage in eggs. Virus preparations were diluted to 1 ml into PBS buffer containing 3% (wt/vol) BSA (PBS-BSA) to HA titers of 256–512. Virus suspensions were applied to slides and the slides were incubated in a closed container and subjected to gentle agitation for 1 h. Unbound virus was washed off by dipping slides sequentially in PBS with 0.05% Tween-20 (PBS-T) and PBS. While still wet, slides were overlaid with corresponding primary antibodies diluted in PBS-BSA, either goat antiserum A/FPV/Rostock/34 (H7N1) (1:500) (for NL/219, NL/230, and Ck/

Conn viruses), ferret anti-A/Canada/444/04 (H7N3) (1:500) (for Can/504 and Can/444 viruses), ferret anti-A/Turkey/VA/4529/02 (H7N2) (1:500) (for Tky/VA virus), ferret anti-A/NY/107/03 (H7N2) (1:500) (for NY/107 virus), chicken anti-A/Rhea/NC/39482/93 (H7N1) (1:500) (for Rhea/NC virus), or sheep anti-A/Vietnam/1203/04 (H5N1) (1:1,000) (for HK/486 virus) (1 h). Slides were washed briefly with PBS-T/PBS as above followed by application of the appropriate secondary antibody conjugates, either anti-ferret-IgG FITC (1:200), anti-goat-IgG FITC (1:200), goat anti-chicken-IgY-FITC (1:200) (Genway Biotechnology), or anti-sheep-IgG-FITC (1:200) in PBS-BSA were subsequently incubated (1 h) followed by PBS-T/PBS washes and a final wash step in deionized water. After the slides were dried in a steam of nitrogen, they were immediately scanned (ProScanArray HT slide scanner with Autoloader, Perkin–Elmer) followed by image analysis with ImaGene 6.1 software (Biodiscovery).

Ferret Transmission Experiments. Male Fitch ferrets, 7–10 months of age (Triple F Farms) and serologically negative by HI assay for currently circulating influenza A H1N1, H3N2, and B viruses were used in this study. Ferrets were housed for the duration of each experiment in a Duo-Flo Bioclean mobile clean room (Lab Products). Ferrets were inoculated with 10^7 EID_{50} of each virus, and nasal washes were collected on indicated days p.i. as described (2). Respiratory droplet and contact transmission experiments were conducted as described (21), with a total of six ferrets used for each experiment.

Hemagglutination Assays. Convalescent sera were collected from all ferrets on days 18–21 p.i./p.c. and tested for H7 specific antibodies by HI by using homologous virus and 1% horse RBCs as described (55). Hemagglutination assays using resialyated turkey RBC were performed as described (56, 57) with minor modifications. Turkey RBC were enzymatically desialyated, followed by resialylation using either α2–6-(*N*)-sialyltransferase (Japan Tobacco) or α2–3-(*N*)-sialyltransferase (Calbiochem). Assays were performed by using both 4 and 8 hemagglutination units of virus yielding identical results.

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研究報告の概要

カナダにおいて、Saffold ウイルスに関連するカルジオウイルス分離株が呼吸器症状を有する 3 名の子供の鼻咽頭吸引物から検出された。Can112051-06 分離株のポリプロテイン配列は、Saffold ウイルスと 91.2%の aa 同一性を有した。しかし、ウイルス表面の EF 及び CD のループは、かなり異なっていた。

ピコルナウイルス科は 9 つの属に分類され、エンテロウイルス、ヘパトウイルス、ライノウイルス、コブウイルス及びパレコウイルスはヒトに感染するが、アフトウイルス、エルボウイルス、テスコウイルス及びカルジオウイルスは動物の病原体である。カルジオウイルス属は、タイラーウイルスと脳心筋炎ウイルス（EMCVs）の 2 つの種に分けられる。ラットやマウスは EMCVs の自然宿主であるが、これらのカルジオウイルスはこれまで、ブタ、げっ歯類、ゾウ、マカク及びヒトに感染することがわかっている。最近、Saffold ウイルス（SAF-V）と暫定的に名付けられた新規カルジオウイルスが、発熱した 8 歳の女子の排便サンプルから分離された。このウイルスは、新規のカルジオウイルスの種と考えられ、知られている他のカルジオウイルスよりも遺伝的にタイラー様ウイルスに関連していた。
SAF-V と、今回の研究で示した SAF-V に関連するカナダの株が、新種のヒトカルジオウイルスの種と分類されるべきか、あるいはタイラーウイルス種の中の新しいクレードとして分類されるべきかについて、今後決定される必要がある。

報告企業の意見	今後の対応
新規の Saffold ウイルスに関連するカルジオウイルス分離株が呼吸器症状を有する 3 名の子供の鼻咽頭吸引物から検出されたとの報告である。 カルジオウイルスは、ピコルナウイルス科に属する直径 22～30nm のエンベロープを有しない RNA ウイルスである。血漿分画製剤からのカルジオウイルス感染に関する報告は、入手していない。 万一、添加剤のアルブミンの原料血漿にカルジオウイルスが混入したとしても、EMCおよびCPVをモデルウイルスとしたウイルスバリデーション試験成績から、製造工程において十分に不活化・除去されると考えている。	新規カルジオウイルスに関する追加情報の入手に努める。

使用上の注意記載状況・その他参考事項等

感染症に関連する記載はない。

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New Saffold Cardioviruses in 3 Children, Canada

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In Canada, cardiovirus isolates related to Saffold virus were detected in nasopharyngeal aspirates from 3 children with respiratory symptoms. Polyprotein sequence of the Can112051-06 isolate had 91.2% aa identity with Saffold virus; however, EF and CD loops of the viral surface varied substantially.

The family *Picornaviridae* contains 9 genera: *Enterovirus, Hepatovirus, Rhinovirus, Kobuvirus,* and *Parechovirus* infect humans, whereas *Aphtovirus, Erbovirus, Teschovirus,* and *Cardiovirus* are animal pathogens (*1*). The genus *Cardiovirus* is divided into 2 species: Theiler viruses and the encephalomyocarditis viruses (EMCVs) (*2–5*). Although rats and mice are the natural hosts for EMCVs, these cardioviruses have been found to infect many animal species including pigs, rodents, elephants, macaques, and humans (*6–9*). Recently, a new cardiovirus provisionally named Saffold virus (SAF-V) was isolated from a stool sample of an 8-month-old girl with fever (*10*). This virus is believed to constitute a novel cardiovirus species and is more genetically related to Theiler-like virus than to other known cardioviruses (*10*). We report the identification and characterization of 3 SAF-V isolates recovered from children with respiratory symptoms.

The Patients

The first patient was a 23-month-old girl who was referred on March 6, 2006, to a tertiary hospital for bilateral otitis media that had not responded to amoxicillin or later to cefprozil. She also had cough, rhinorrhea, and fever of 39°C. Her 5-month-old brother had similar clinical signs. Blood cultures were negative, as were antigen detection tests for influenza A and B viruses, the respiratory syncytial virus, and adenoviruses. After 24 hours, the girl was discharged with a diagnosis of bilateral acute otitis media secondary to a viral infection. A nasopharyngeal aspirate collected at the time of admission was inoculated onto different continuous cell lines including human lung adenocarcinoma (A-549); human rhabdosarcoma (RD); transformed human kidney (293); human colon adenocarcinoma (HT-29); human laryngeal carcinoma (Hep-2); human foreskin fibroblast; mink lung; and Vero, MDCK, and rhesus monkey kidney (LLC-MK2) cells. Cultures were incubated for 3 weeks at 37°C in 5% CO_2. A viral isolate (Can112051-06) with cytopathic effects (round cells) suggestive of a picornavirus was observed only in LLC-MK2 cells after 6 days of incubation (Figure 1). An immunofluorescent assay that used the Pan-Enterovirus Blend kit (Light Diagnostics, Levingston, UK) gave a moderate fluorescent signal. Nucleic acid extracts from Can112051-06 were further analyzed with a multiplex real-time reverse transcription–PCR (RT-PCR) assay for common respiratory viruses (influenza A and B viruses,

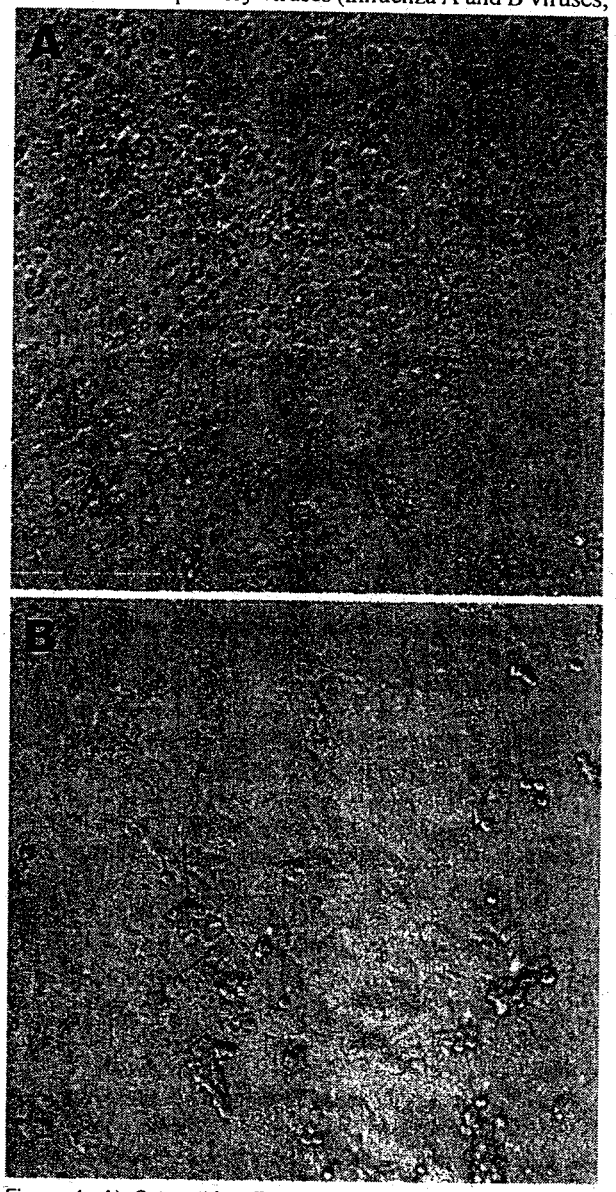

Figure 1. A) Cytopathic effects (round cells) observed 6 days after infection of rhesus monkey kidney (LLC-MK2) cells (second passage) with the Can112051-06 Saffold virus–like cardiovirus strain. B) Uninfected LLC-MK2 cells. Magnification ×10.

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human respiratory syncytial virus, and human metapneumovirus) (*11*) as well as RT-PCR assays for enteroviruses and parechoviruses (*12*); results were negative.

The supernatant from LLC-MK2–infected cells was treated with DNase and divided into 2 aliquots for DNA and RNA extractions by using the QIAamp Blood Mini Kit and QIAamp Viral RNA extraction kits (QIAGEN, Mississauga, Ontario, Canada), respectively. Nucleic acids were then used in the sequence-independent single-primer amplification method as described (*13*). Amplicons of 800–1,200 bp obtained from RNA samples were cloned and sequenced.

Sequence determination of cloned amplicons followed by tBLASTx analysis showed similarity of Can112051-06 sequences with the SAF-V VP4 and 2C sequences (data not shown). Subsequent PCR amplifications and sequencing reactions that used primers selected from our clones and the complete SAF-V genome sequence (GenBank accession no. EF165067) enabled us to determine the complete polyprotein encoding region of the Can112051-06 isolate (GenBank accession no. AM922293). This region was 6,879 nt long compared with 6,888 nt for the SAF-V polyprotein sequence; nucleotide identity between the 2 strains was 82.5%. The Can112051-06 polyprotein comprised 2,293 aa compared with 2,296 aa for the SAF-V polyprotein; amino acid identity between the 2 strains was 91.2%. Deletions of 1 aa in the VP2 and 2 in the VP1 proteins were found in Can112051-06 with regard to the prototype SAF-V strain. As expected, the Can112051-06 and SAF-V polyproteins contained 11 putative cleavage sites. The 8 aa flanking these sites were conserved; 6 sites were identical in the 2 strains, whereas the remaining sites had 1- or 2-aa differences (Table 1). The resulting 12 proteins of Can112051-06 and SAF-V had 76.1%–100% aa identities (Table 2). The highest difference level was seen in the L peptide. In addition to the L peptide, some cardioviruses, in particular Theiler's murine encephalomyelitis virus strains that are associated with persistent infections, contain an alternate open reading frame (ORF), the so-called L* (*14*). As for the prototype SAF-V strain, the Can112051-06 putative L* ORF is unlikely to encode a protein because it has an ACG (instead of ATG) start codon (data not shown). In addition, contrasting with the SAF-V L*, which contained 57 aa, the Can112051-06 L* sequence contained only 34 aa. Comparison of the L* sequence of Can112051-06 with the first 34 aa of the SAF-V L* sequence showed 60.6% identity (data not shown). Four small loops are exposed on the virion surface of cardioviruses; 2 are part of the VP2 EF loop structure, and 2 are part of the VP1 CD loop structure. The EF loop structure of Can112051-06, which contained 55 aa (residues 274-328 of the polyprotein), had 61.8% aa identity with that of SAF-V (Figure 2, panel A). Similarly, the CD loop structure of Can112051-06, which contained 40 aa (residues 712-751 of the polyprotein), had 67.5% aa identity with the SAF-V counterpart (Figure 2, panel B).

Table 1. Cleavage sites of Can112051-06 and prototype Saffold virus cardiovirus polyproteins*

Cleavage site	Can112051-06	Saffold virus
L / VP4	MEPQ / GNSN	MEPQ / GNSN
VP4 / VP2	PLLM / DQNT	PLLM / DQNT
VP2 / VP3	LEDQ / SPIP	LEAD / SPIP
VP3 / VP1	YTPH / GVDN	YTPQ / GVDN
VP1 / V2A	LELQ / NPIS	LELQ / DPIS
2A / 2B	FQLQ / GGVL	FQLQ / GGVL
2B / 2C	LQQQ / SPVR	LQQQ / SPIR
2C / 3A	LVAQ / SPGN	LVAQ / SPGN
3A / 3B	EGEQ / AAYS	EGEQ / AAYS
3B / 3C	LDVQ / GGGK	LDVQ / GGGK
3C / 3D	LIPQ / GAIV	LTPQ / GAIV

*Can112051-06 GenBank accession no. AM922293; Saffold virus GenBank accession no. EF165067.

Other respiratory samples with picornavirus-like cytopathic effects on LLC-MK2 cells and weakly immunofluorescent signals according to the Pan-Enterovirus Blend Kit were screened for cardiovirus SAF-V by using a specific RT-PCR assay targeting a 2A–2C encoding region (1,407 nt, 469 aa). With use of this strategy, 2 more cases were noted in September 2006: 1 in a 19-month-old child hospitalized for suspected bacteremia and a cold and 1 in a 4-year-old child hospitalized for right lung pneumonia and otitis media. The 2A-2C aa sequences of these additional isolates were identical and shared 96.6% and 97.2% aa identities with the corresponding regions of Can112051-06 and the prototype SAF-V, respectively.

Conclusions

Our findings suggest a pathogenic role for SAF-V-like viruses in humans. Although the polyprotein sequences of the Can112051-06 strain and the original US strain were related, the EF and CD loop structures varied substantially (61.8% and 67.5% aa identities, respectively). For com-

Table 2. Amino acid identities between Can112051-06 and prototype Saffold virus proteins*

Protein	% Identity
L	76.1
VP4	97.2
VP2	83.9
VP3	85.2
VP1	76.7
2A	95.8
2B	97.6
2C	96.6
3A	100
3B	95.0
3C	96.8
3D	97.0

*Can112051-06 GenBank accession no. AM922293; Saffold virus GenBank accession no. EF165067.

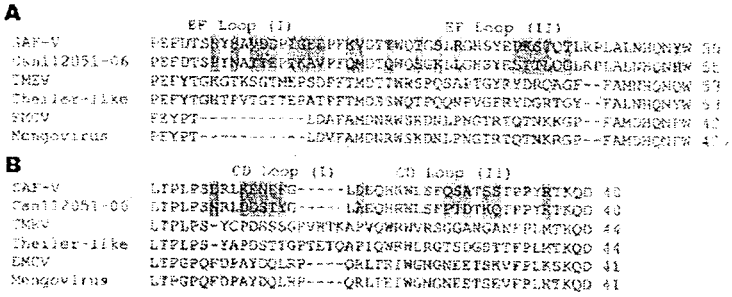

Figure 2. Comparison of amino acid sequences of the A) EF loop structure (part of the VP2 protein) and B) the CD loop structuré (part of the VP1 protein) between Can112051-06 and other cardioviruses including Saffold virus (SAF-V), Theiler's murine encephalomyelitis virus (TMEV), Theiler-like virus, encephalomyocarditis virus (EMCV), and Mengovirus. Amino acid differences between Can112051-06 and SAF-V are shaded.

parison, the EF and CD loop structure sequences of EMCV and Mengovirus (2 members of the EMCV species) have 95.2% and 95.1% aa identities, respectively. The difference between time of isolation of SAF-V (1981) and the Can112051-06 strain (2006) is unlikely to be responsible for such a high level of sequence variation. We previously showed that the amino acid sequences of the VP0-VP1 capsid region of Canadian human parechovirus 1 strains isolated from 1985 through 2004 had 89.2% to 97.5% identities (*12*). Because the EF and CD loop structures are exposed on the viral surface of cardioviruses and thus constitute an important site for recognition by neutralizing antibodies (*15*), Can112051-06 and the original SAF-V might represent different serotypes, although further serologic studies are needed to confirm this hypothesis. The implication of the weak immunofluorescent signal seen in cardiovirus-infected cells stained with an enterovirus antibody is uncertain because of the considerable difference between the capsid proteins of cardioviruses and enteroviruses, which constitute 2 separate picornavirus genera.

In contrast to the initial recovery of this virus from a stool sample (*10*), our 3 strains were recovered from nasopharyngeal aspirate samples of children with fever and some other respiratory signs. The cardioviruses were the only pathogens identified in these samples. Whether SAF-V and the related Canadian strains described in this study should be classified as a new human *Cardiovirus* species or as a new clade within the *Theilovirus* species remains to be determined.

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Dr Boivin is the holder of the Canada Research Chair on Emerging Viruses and Antiviral Resistance at Laval University. His research interests include antiviral resistance mechanisms for herpes and influenza viruses and characterization of emerging viruses.

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