表4　09-2130株、09-2272株間のFタンパク質のアミノ酸置換

ORF	aa	2272	2130
F	8	L	I
	240	A	T
	321	N	S
	493	K	T
	526	I	V
	532	D	N
	546	K	R

表5　09-2130株、09-2272株間のHNタンパク質のアミノ酸置換

ORF	aa	2272	2130
HN	3	E	G
	22	A	I
	23	N	D
	34	H	Y
	42	A	T
	46	T	A
	70	T	I
	187	S	T
	245	I	V
	256	K	R
	279	T	I
	332	D	N
	355	S	K
	356	K	S
	385	R	H
	443	R	K

	450	T	A
	453	R	K
	489	V	F
	511	N	S
	514	E	K
	525	K	Q
	558	F	L
	570	V	I

図1　緑色蛍光タンパク発現組換えマウスパラインフルエンザウイルス１型のVero細胞ならびにVero/TMPRSS2細胞での増殖

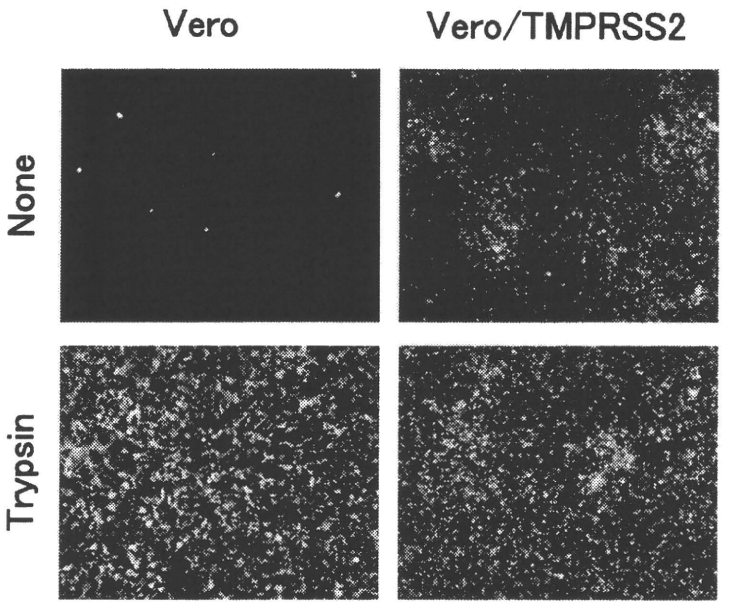


図2　Vero細胞ならびにVero/TMPRSS2細胞でのプラーク像

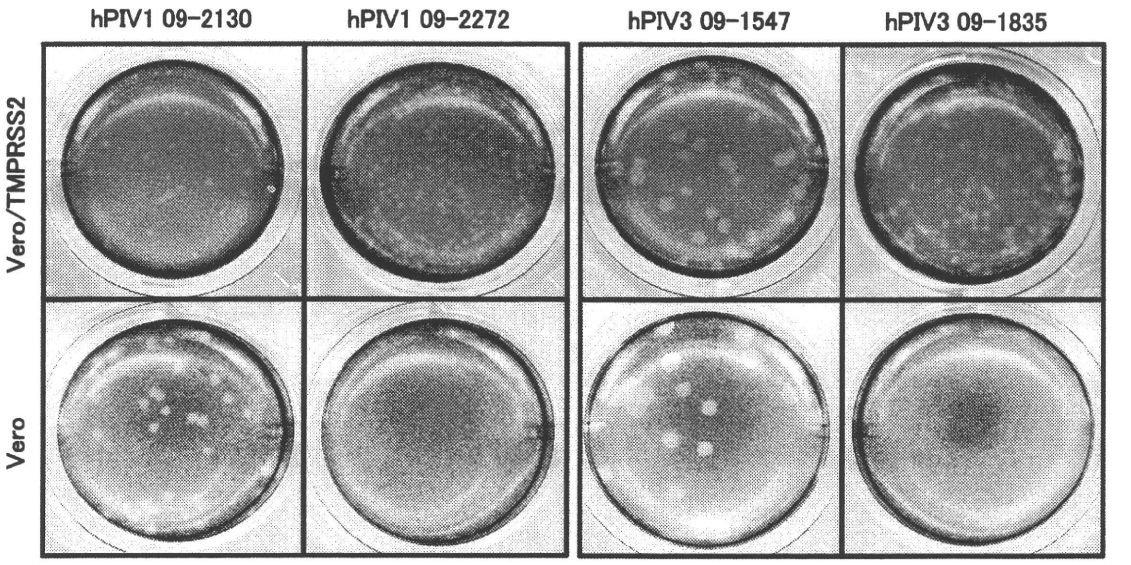


図3　ヒトパラインフルエンザウイルス１型Ｆタンパク質の立体構造モデルと09-2130株、09-2272株間のアミノ酸置換部位

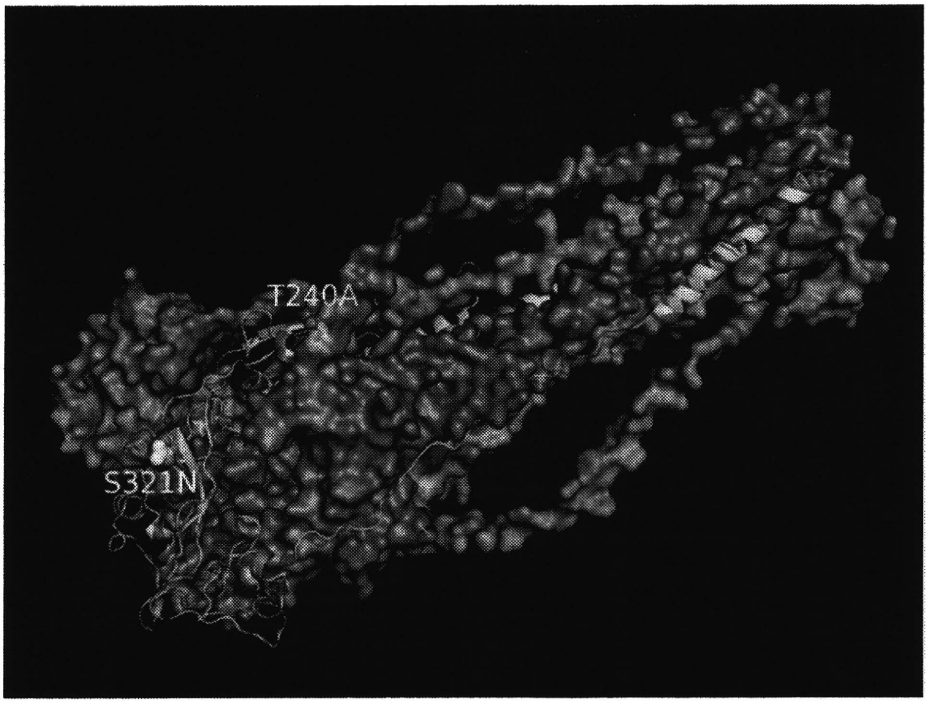
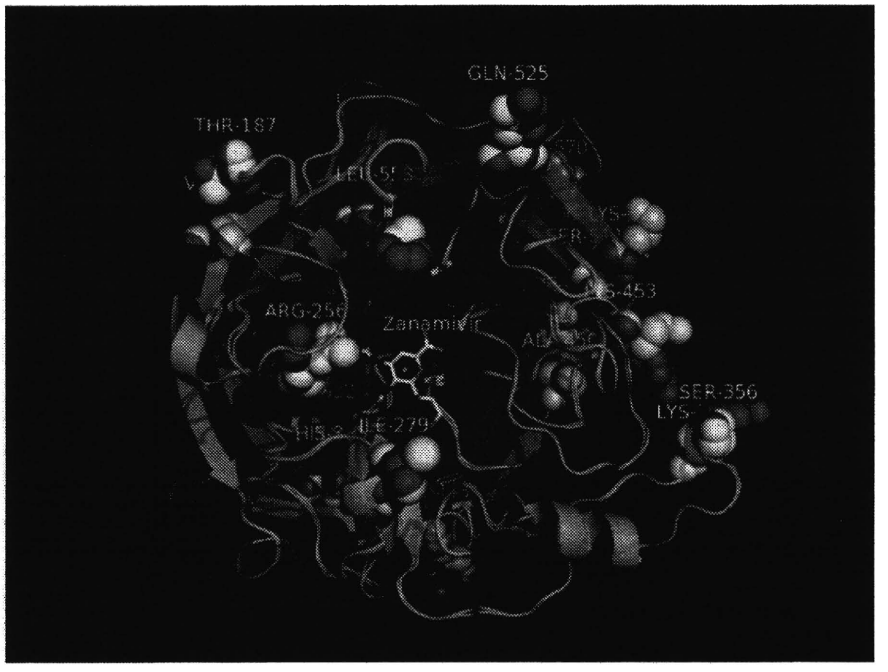


図4　ヒトパラインフルエンザウイルス１型HNタンパク質の立体構造モデルと09-2130株、09-2272株間のアミノ酸置換部位



厚生労働科学研究費補助金(新型インフルエンザ等新興・再興感染症研究事業)
分担研究報告書

呼吸器感染ヒトコロナウイルス全般の感染機序解明、分離技術の向上・制御に関する研究

研究分担者　松山州徳　ウイルス第三部

研究要旨

SARS コロナウイルス及びインフルエンザウイルス、メタニューモウイルスは肺に存在するプロテアーゼ TMPRSS2 を利用して極めて効率よく感染することが報告されている。今回我々は、病原性の異なる数種のヒトコロナウイルス（SARS、NL63、229E）を用い、TMPRSS2 感受性の比較、及び、市販のプロテアーゼ阻害剤による感染阻止効果を調べ、これらのウイルスはいずれも TMPRSS2 を利用して細胞に侵入すること、及びセリンプロテアーゼ阻害剤とシステインプロテアーゼ阻害剤の同時に処理により、細胞への感染を完全に阻止できることを明らかにした。

A. 研究目的

肺に特異的に存在する膜貫通型セリンプロテアーゼ(TMPRSS2)の発現細胞に、コロナウイルスは極めて効率よく感染する。この細胞はコロナウイルスの分離技術やサーベイランスの向上に寄与できるとともに、感染機序の解明及びウイルス細胞侵入阻害薬のスクリーニングにも利用できる可能性があり、それを検証する。さらに、細胞膜貫通型プロテアーゼとレセプター及びS蛋白の相互作用、蛋白の構造解析を詳しく調べ、ウイルス感染阻害に重要な領域を見つけ出し、将来のワクチン開発や抗ウイルス剤開発に繋げていく。

B. 研究方法

VSV シュードタイプウイルスの表面に、それぞれのコロナウイルス（SARS、NL63、229E 及び 229E の臨床分離株）の S 蛋白をもたせたものを作成し、細胞侵入実験に用いた。これらのシュードタイプウイルスは感染細胞で GFP を発現するので、容易に感染価を調べることができる。TMPRSS2 発現細胞と非発現細胞に感染させ、TMPRSS2 感受性の比較をおこなった。また市販のプロテアーゼ阻害剤で処理した細胞へのウイルスの感染を調べ、TMPRSS2 の活性阻害剤の検索をおこなった。

C. 研究結果

SARS と NL63 のシュードタイプウイルスの TMPRSS2 発現細胞への感染価は非発現細胞と比較して、３倍から５倍高くなっていた。この結果は TMPRSS2 が ウイルスを活性化し細胞侵入を促進させることを示唆している。また 229E では TMPRSS2 発現細胞で１．５倍程度感染価が高くなったが、臨床分離 229E ではさらに３倍程度高くなることがわかった。この結果は、229E はヒトの生体内では TMPRSS2 を利用して感染する傾向が高いことを示唆している。また、セリンプロテアーゼ阻害剤（Camostat）とシステインプロテアーゼ阻害剤（E64d）を同時に作用させることにより、TMPRSS2 発現培養細胞へのこれらのコロナウイルス侵入を完全に阻止できることも明らかにした。

D. 考察

今回用いた TMPRSS2 発現細胞は、我々が人

工的に作成したものであり、実際の肺での現象を反映しているのかどうかは不明である。今後、実際の肺に性質が近いと考えられる、ヒト肺胞上皮初代培養を用いて同様の実験をおこなうことにより、肺由来細胞ではどのプロテアーゼが感染に利用され、どのようなプロテアーゼ阻害剤の組み合わせにより、感染を阻止できるのかを明らかにしたい。

E. 結論

ヒトコロナウイルスは肺特異的プロテアーゼ TMPRSS2 を利用して細胞に侵入すること、及びセリンプロテアーゼ阻害剤とシステインプロテアーゼ阻害剤の同時処理により、細胞への感染を完全に阻止できることを明らかにした。

F. 健康危険情報

なし

G. 研究発表

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H. 知的財産権の出願・登録状況

なし

厚生労働科学研究費補助金（新型インフルエンザ等新興・再興感染症研究事業）
分担研究報告書

新規ワクチン開発と感染細胞モデル系の構築に関する研究

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研究要旨

Respiratory syncytial virus (RSV)、parainfluenza virus (PIV)、Human Rhinovirus(HRV)による呼吸器ウイルス感染は小児や老人における下気道炎の主原因となる。さらに、これらのウイルス感染は、感染喘息の引き金や増悪因子となることが明らかとなりつつある。しかしながら、これらのウイルス感染症に対する有効なワクチンは開発されていない。また、病原体分離や阻害剤スクリーニングのための優れた培養細胞モデル系は構築されていない。我々はPIV-3 ワクチン開発に向け、コムギ無細胞系を用いたリコンビナントウイルス外被タンパク質（PIV-3-HN）のベクター構築および機能的タンパク質の大量精製を行った。さらに、広いウイルス感染トポロジーを有する培養細胞の樹立を目指し、ヒト胎児肺線維芽細胞 MRC-5 の不死化を行いこれに成功した。加えて、サル皮膚線維芽細胞から幹細胞を誘導することで呼吸器ウイルス感染感受性を示す培養細胞の樹立を試みた。

A. 研究目的

呼吸器ウイルスは、ある一定の割合で気管支炎や肺炎などの重症呼吸器感染症を引き起こすことがよく知られており、重症呼吸器感染症(肺炎)による年間死亡者数は近年増加している。しかしながら、インフルエンザウイルスを除く呼吸器ウイルス感染症に対して有効なワクチンが開発されていない。特に乳幼児や老人に対して重篤な下気道感染を引き起こすRSVやPIV-3型に対するワクチン開発の基礎研究を進めることは重要である。今回、PIV-3 ワクチン開発に向け、コムギ無細胞系を用いたリコンビナントウイルス外被タンパク質（PIV-3-HN）の大量精製を行い、新規ワクチン抗原として有効性について評価を行う。また、DNA ワクチンを開発する目的で、哺乳動物コドン最適化 PIV-3-HN 発現ベクターの構築を行う。

一方、呼吸器ウイルス感染症サーベイランスにおいて約２／３がその原因ウイルスの分離に至っておらず、広い感染トポロジーを有する培養細胞の樹立が必須である。今回、既存の呼吸器ウイルス感染感受性正常細胞（MRC-5）の不死化を試行すると同時に、細胞初期化技術による新規呼吸器ウイルスに感受性を有する培養細胞モデル系の樹立を試みた。

B. 研究方法

1. コムギ無細胞系を用いたPIV-3-HN合成

コムギ無細胞系発現ベクターpEU-E01-GST-PIV3HNを構築し、コムギ無細胞タンパク質合成系を用い、PIV-3-HNタンパク質全長タンパク質の合成を行った。精製はグルタチオンビーズによるカラム法を用いた。GST-PIV3HN間の切断はTEVプロテアーゼを用いて行った。また、DNA ワクチンへの応用を考慮し、動物細胞コドンの最適化した PIV-3-HN の全長 cDNA を合成した。

2. ヒト胎児肺線維芽細胞 MRC-5 細胞不死化

正常細胞の不死化を誘導するレンチウイルスベクターとしてpLenti6_TERT, pLenti6_HPV16-E7, pLenti6_sh-p16_TetOff_FRT-GFP-BSD を作成した。作成したベクターを用い、MRC-5/TERT 細胞に不死化誘導因子を導入した。

3. 呼吸器ウイルス感染感受性アカゲザル細胞モデル系の構築

アカゲザル皮膚線維芽細胞に細胞初期化因子導入後、Embryoboid body 形成法を用いて細胞分化を行った。ウイルス感受性試験ではRSV およびPIV3 をMOI=1 で標的細胞に感染させ、48 時間後に細胞障害性および合胞体の形成を観察した。また感染細胞からRNA を抽出し、RT-PCR を用いてウイルス遺伝子の発現レベルを測定した。

C. 研究結果

1. PIV-3-HN 全長タンパク質を用いた新規ワクチンの開発

真核生物型無細胞タンパク質合成技術であるコムギ胚芽タンパク質合成システムを用いてリコンビナント PIV-3-HN 全長タンパク質の作成を試みた。無細胞合成用の pEU-E01-GST ベクターに PIV3-HN 全長 cDNA をサブクローニングした。次に全自動コムギ無細胞系タンパク質合成装置 PROTEMIST-DT-II を用いて GST 融合 PIV-HN タンパク質の大量合成を行った。合成したGST 融合PIV-HN タンパク質をグルタチオンセファロースビーズ法を用いて回収し、TEV プロテアーゼを用いてGST タグを切断した。本法により一度の合成で約200μg の精製タンパク質が作製できる。今後は本標品と核酸アジュバント混合剤を BALB/c マウスに投与し、ワクチン抗原としての有効性を検討する予定である。また、DNA ワクチンを用いた Prime-boost 法への応用として PIV-3-HN をマウスコドンに最適化した発現ベクターを構築した。本標品を上記のマウスワクチンモデルにおいて評価を行うとともに、PIV3 感染を阻止する中和抗体の開発を行う予定である。

2. 呼吸器ウイルス感染感受性細胞 MRC-5 の不死化

正常細胞の不死化を誘導するレンチウイルスベクター、pLenti6_TERT, pLenti6_HPV16-E7, pLenti6_sh-p16_TetOff_FRT-GFP-BSD を作成した。作成したウイルスベクターを MRC-5/TERT 細胞に感染させ、通常培養法にて長期培養を行った。現段階で少なくとも 100 回以上の分裂が可能な MRC-5/TERT, MRC-5/TERT_sh-p16, MRC-5/TERT_HPV16-E7 の 3 つの不死化 MRC-5 細胞の誘導に成功した。今後は本細胞群を用いて広範囲な呼吸器ウイルスの感染性や細胞障害性について考察する予定である。

3. 新しい呼吸器ウイルス感染感受性培養細胞モデル系の構築 (Rhesus Monkey Stem Cell : rSC)

広いウイルス感染トポロジーを有する細胞を樹立する目的で、アカゲザル皮膚線維芽細胞に細胞初期化因子導入し、Rhesus Monkey Stem Cell (rSC) を作成した。ウイルス感染感受性試験により、rSC は RSV と PIV-3 に感染し、多核細胞や合胞体を形成することが確認された。また、感染細胞から RNA を抽出し、RSV G 遺伝子および PIV-3 HN 遺伝子領域の RT-PCR を行ったところ、ウイルス転写産物を確認した。今後は通常培養法への馴化を行うとともに、各種呼吸器ウイルスへの感染感受性や感染粒子形成能等について詳細に検討を行う。また、幹細胞とウイルス感染の関連について分子細胞生物学的観点から考察を行う予定である。

D. 考察

今回、我々は呼吸器ウイルス抗原タンパク質の合成にコムギ無細胞タンパク質合成系を活用した。大腸菌や培養細胞などの生細胞を基盤とする従来の遺伝子発現方法には，生産できるタンパク質の分子種，量等に限界があり、特に細

胞毒性を有するウイルスタンパク質の合成は難しかった。また、タンパク質の立体構造上の問題等のワクチン抗原としての品質の問題も多分にあった。無細胞タンパク質合成法は，生体の遺伝情報発現系を人工容器内に取り揃え，遺伝子 DNA からタンパク質を鋳型合成する試験管内合成法であるため、これらの問題が全て解決できている。その中でも、コムギ無細胞系は真核生物のマルチドメインタンパク質の合成に優れた性能を発揮し、特にウイルスタンパク質のエピトープ領域の合成に優れている。高効率コムギ無細胞タンパク質合成法の基盤として、（1）高翻訳促進能を有する mRNA の5'末端及び3'末端非翻訳配列の設計、（2）専用高発現ベクターの構築、（3）新規改良 PCR 法を用いる転写鋳型構築法、(4)高効率翻訳反応方法、などのキー技術があげられる。これらはワクチン抗原としてのウイルスタンパク質の合成法として優れていると考えられる。

また、今回我々は新規のウイルス感染細胞モデルとして呼吸器ウイルス感染感受性細胞である MRF-5 細胞の不死化および新規アカゲザル細胞株の樹立に成功した。今後はこれらの細胞株のウイルス感受性のプロフィールを確認するとともに、ウイルス複製や病原性発現に至る分子基盤について解析を行う予定である。

E. 結論

今回我々は PIV-3 ワクチン開発に向け、コムギ無細胞系を用いたリコンビナントウイルス外被タンパク質（PIV-3-HN）のベクター構築および機能的タンパク質の大量精製に成功した。また、病原体分離や阻害剤スクリーニングのための優れた培養細胞モデル系は構築を目指しヒト胎児肺線維芽細胞 MRC-5 の不死化を行いこれに成功した。また、アカゲザル皮膚線維芽細胞から幹細胞を誘導することで呼吸器ウイルス感染感受性を示す培養細胞を樹立した。

F. 研究発表

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G. 知的所有権の取得状況

1) 「幹細胞の安定性維持、複製を制御するためのペプチジルプロリルイソメラーゼＰｉｎ１の利用」 特願:2010-238548 出願日: 平成 22 年 10 月 25 日 出願人: 公立大学法人横浜市立大学 発明者: 梁 明秀、西 真由子

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Phylogenetic and cluster analysis of human rhinovirus species A (HRV-A) isolated from children with acute respiratory infections in Yamagata, Japan

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ABSTRACT

We performed phylogenetic and cluster analysis of human rhinovirus species A (HRV-A) isolated from 76 children with acute respiratory infection in Yamagata prefecture, Japan during the period 2003–2007. Phylogenetic trees based on the nucleotide and amino acid sequences of the *VP4/VP2* coding region showed that the present strains could be classified into 11 and 8 clusters, respectively. The homology among the present strains ranged from 66.6% to 100% at the nucleotide level and 84.7% to 100% at the amino acid level. The interspecies distance (mean ± standard deviation) was calculated to be 0.235 ± 0.048 at the nucleotide level and 0.076 ± 0.033 at the amino acid level. In addition, the phylogenetic trees created based on the nucleotide and amino acid sequences showed that HRV-A strains belonging to some clusters were associated with both upper respiratory infection and wheezy bronchiolitis, while other strains were associated with upper respiratory infection alone. These results suggest that the present HRV-A isolates had a wide nucleotide divergence and were associated with acute respiratory infection, including upper respiratory infection and wheezy bronchiolitis, in Yamagata prefecture, Japan during the investigation period.

1. Introduction

Human rhinoviruses (HRVs), which are positive sense-stranded RNA viruses belonging to the *Picornaviridae* family, cause acute respiratory infection (ARI) in humans and are the major pathogen for the common cold (Turner and Couch, 2007). HRV, the major pathogen for the common cold, is responsible for around 50% of asthma exacerbations and is one of the factors that can direct the infant immune system towards an asthmatic phenotype (Nicholson et al., 1993; Johnston et al., 1996; Jackson et al., 2008). In addition, accumulating evidence suggests that some patients with asthma or exacerbation of asthma may be associated with HRV infection, although the pathophysiology of this association is not clear at present (Wos et al., 2008). Thus, HRV is being reevaluated as an important agent of ARI in humans (Papadopoulos et al., 2002; Imakita et al., 2000; Wos et al., 2008).

More than 100 serotypes of HRV have been confirmed, and these viruses have been classified into many clusters on the basis of genetic analysis (Savolainen et al., 2002a,b). Recently, it was reported that HRV can be classified into 3 species (HRV-A, -B, and -C) on the basis of phylogenetic analysis of the *VP4* gene (Lau et al., 2007). Strains belonging to HRV-A and -B species are culturable and have been implicated as the most prevalent viruses associated with ARI (Savolainen et al., 2002a), whereas those of the HRV-C species are not culturable. However, the genetic characteristics and epidemiology of domestic HRV-A infection are not known. Therefore, as a general indicator of domestic infection, we performed phylogenetic and cluster analysis of HRV-A isolated from patients

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with ARI in Yamagata prefecture, Japan during the period 2003–2007.

2. Materials and methods

2.1. Isolates and patients

We analyzed 76 HRV-A isolates obtained from patients with upper respiratory infection (URI) (70 patients) or wheezy bronchiolitis (6 patients) referred to pediatric clinics in Yamagata prefecture during the period 2003–2007. The samples were obtained by the local health authority of Yamagata prefecture for the surveillance of viral diseases in Japan. We diagnosed URI, also known as the common cold, and URI that typically affects the upper airway which includes the nose (sinusitis), throat (pharyngitis), and voice box (laryngitis) (Cherry, 2003). Wheezy bronchiolitis was diagnosed due to the presence of wheezing alone or chest retractions in association with a URI (Robert, 2003). A detailed listing of patients and isolates is provided in Table 1. All patients were under 16 years of age (5.8 ± 3.3 years; mean ± standard deviation [SD]). No significant seasonal variation of HRV-A isolation was seen in this study.

2.2. Cell culture and virus isolation

To isolate various respiratory viruses, we used HEF, HEp-2, Vero E6, MDCK, RD-18S and GMK cells in this study. Cells were grown in Eagle's minimal essential medium (Nissui No. 3; Nissui Pharmaceutical Co., Tokyo, Japan) containing 5–10% fetal bovine serum or calf serum at 37 °C in a humidified atmosphere of 5% CO_2 (Mizuta et al., 2008). Cells were grown in 96 well microplates (Greiner Bio-One, Frickenhausen, Germany).

After the plates were washed with phosphate-buffered saline without calcium and magnesium (PBS), 100 μL of maintenance medium was added to each well of the plates. Each specimen was then centrifuged at 3000 rpm for 15 min and 75 μL of the supernatant was inoculated into 2 wells of each cell line. The inoculated plates were incubated at 33 °C in a CO_2 incubator. We observed the plates two or three times per week for cytopathic effects (CPEs) over 14 days for all cell lines, without passage or medium change (Mizuta et al., 2008). When a suspected HRV CPE was observed, we passaged and stored the cells at −80 °C until analysis. Viral identification was carried out by reverse-transcription polymerase chain reaction (RT-PCR) and sequence analysis (Savolainen et al., 2002a).

2.3. RNA extraction, RT-PCR, and sequencing

For RNA extraction, RT-PCR, and sequence analysis, infected culture fluids were centrifuged at 3000 × *g* at 4 °C for 15 min and the supernatants were used for RT-PCR and sequence analysis as described previously (Savolainen et al., 2002a; Iwai et al., 2006). Briefly, viral RNA was extracted from 140 μL supernatant with a QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA). The RT-PCR procedure was performed according to the manufacturer's instructions (Access RT-PCR System; Promega, Madison, WI). The primers for RT-PCR were as follows: 5′ GGG ACC AAC TAC TTT GGG TGT CCG TGT 3′ (9895 forward; sense [534–560 nt]), and 5′ GCA TCI GGY ARY TTC CAC CAC CAN CC 3′ (9565 reverse; antisense [1083–1058 nt]). The following protocol was used: 45 min at 48 °C (reverse transcription), 2 min at 94 °C (denaturation), and 40 cycles of 94 °C for 30 s, 60 °C for 1 min, and 68 °C for 2 min followed by 7 min at 68 °C in the last cycle for elongation. Purification of DNA fragments and nucleotide sequence determination were performed as described previously (Iwai et al., 2006).

2.4. Phylogenetic analysis and calculation of pairwise genetic distances

For phylogenetic analysis, the nucleotide sequences (positions 623–1012; 390 bp) and deduced amino acid sequences (130 aa) of the partial HRV *VP4/VP2* coding region were analyzed phylogenetically with the CLUSTAL W program available on the DNA Data Bank of Japan homepage (http://www.ddbj.nig.ac.jp/index-j.html) and TreeView (version 1.6.6; http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). Evolutionary distances were estimated according to the Kimura's 2-parameter method, and the phylogenetic tree was constructed with the neighbor-joining method (Kimura, 1980; Saitou and Nei, 1987). The reliability of the tree was estimated with 1000 bootstrap replications. Genetic distances among reference (representative) strains may be important in phylogenetic analysis. Thus, to construct the phylogenetic tree, we preferably selected the reference strains having constant genetic distances (interspecies distances, 0.237 ± 0.030), and used the following as references strains in this study: HRV1B, HRV7, HRV8, HRV9, HRV12, HRV15, HRV16, HRV19, HRV28, HRV29, HRV47, HRV49, HRV55, HRV56, HRV60, and HRV63. In addition, some human rhinovirus species B (HRV-B), human rhinovirus species C (HRV-C) and human enterovirus species D (HEV-D) were used as reference strains as previously described (Piralla et al., 2009). Echovirus 11(Echo-11), which belongs to the human enterovirus species B was used as the outgroup (Piralla et al., 2009). In order to assess interspecies frequency distributions and the frequency distribution of each intercluster of HRV-A, we calculated pairwise genetic distances for all of the strains, including the present isolates and reference strains, as previously described (Katayama et al., 2002).

3. Results

3.1. Phylogenetic and cluster analysis

Phylogenetic trees based on the nucleotide sequences (390 nt) and the deduced amino acid sequences (130 aa) of the *VP4/VP2* coding region, including the present strains ($n = 76$) and representative reference strains ($n = 16$) belonging to HRV-A, are shown in Fig. 1a and b, respectively. In addition, we show the corresponding phylogenetic trees for the representative reference strains alone (Fig. 1c and d, respectively). Phylogenetic trees based on the nucleotide and deduced amino acid sequences for the present and reference strains of HRV-A species could be classified into 11 and 8 clusters, respectively. At the nucleotide level, the homology among the present strains ranged from 66.6% to 100%, while at the deduced amino acid level the homology ranged from 84.7% to 100%. On the phylogenetic tree based on the nucleotide sequences, the numbers of the present strains in each cluster were as follows: Cluster 1, 6 strains; Cluster 2, 8 strains; Cluster 3, 7 strains; Cluster 4, 8 strains; Cluster 5, 10 strains; Cluster 6, 7 strains; Cluster 7, 4 strains; Cluster 8, 4 strains; Cluster 9, 6 strains; Cluster 10, 10 strain; and Cluster 11, 6 strains. On the phylogenetic tree based on the deduced amino acid sequences, the numbers of the present strains in each cluster were as follows: Cluster 1, 20 strains; Cluster 2, 9 strains; Cluster 3, 10 strains; Cluster 4, 1 strain; Cluster 5, 11 strains; Cluster 6, 4 strains; Cluster 7, 19 strains; and Cluster 8, 2 strains. Notably, on the phylogenetic tree based on nucleotide sequences, Clusters 2, 3, 5 and 9 were associated with both URI and wheezy bronchiolitis, whereas other strains were associated with URI alone. As well, on the phylogenetic tree based on amino acid sequences, Clusters 3, 7, and 8 were associated with both URI and wheezy bronchiolitis. These results suggest that the present HRV-A strains which had a wide nucleotide divergence (66.6–100% homology) were circulating in the Yamagata area during the investigation period.

Table 1
Patients' data, HRV strain, and cluster.

Patient	Age (year)	Sampling date	Disease	Strain	Cluster	GenBank accession no.
1	16	23 January, 2003	URI*	HRVi/Yamagata.JPN/4.03	8**, 1***	AB474093
2	3	25 March, 2003	URI	HRVi/Yamagata.JPN/13.03	3, 5	AB474094
3	15	18 April, 2003	URI	HRVi/Yamagata.JPN/16.03	5, 3	AB474095
4	7	18 April, 2003	URI	HRVi/Yamagata.JPN/16-2.03	1, 7	AB474096
5	11	30 April, 2003	URI	HRVi/Yamagata.JPN/18.03	9, 2	AB474100
6	9	30 April, 2003	URI	HRVi/Yamagata.JPN/18-2.03	3, 5	AB474101
7	5	7 May, 2003	URI	HRVi/Yamagata.JPN/19.03	11, 6	AB474102
8	7	12 May, 2003	URI	HRVi/Yamagata.JPN/20.03	10, 6	AB474103
9	1	10 May, 2003	URI	HRVi/Yamagata.JPN/19-2.03	6, 1	AB474104
10	1	12 May, 2003	URI	HRVi/Yamagata.JPN/20-2.03	11, 2	AB474105
11	0	9 September, 2003	URI	HRVi/Yamagata.JPN/37.03	3, 5	AB474106
12	4	20 September, 2003	Wheezy bronchiolitis	HRVi/Yamagata.JPN/38.03	9, 8	AB474107
13	2	22 September, 2003	Wheezy bronchiolitis	HRVi/Yamagata.JPN/39.03	2, 7	AB474108
14	2	30 September, 2003	URI	HRVi/Yamagata.JPN/40.03	10, 1	AB474109
15	6	6 October, 2003	URI	HRVi/Yamagata.JPN/41.03	3, 7	AB475008
16	0	10 October, 2003	URI	HRVi/Yamagata.JPN/41-2.03	3, 7	AB474110
17	0	10 October, 2003	LRI	HRVi/Yamagata.JPN/41-3.03	8, 1	AB474111
18	9	20 October, 2003	URI	HRVi/Yamagata.JPN/43.03	3, 7	AB474112
19	0	12 November, 2003	Wheezy bronchiolitis	HRVi/Yamagata.JPN/46.03	3, 7	AB475009
20	6	17 November, 2003	URI	HRVi/Yamagata.JPN/47.03	10, 2	AB474113
21	2	19 November, 2003	URI	HRVi/Yamagata.JPN/47-2.03	9, 8	AB474114
22	11	20 November, 2003	URI	HRVi/Yamagata.JPN/47-3.03	10, 2	AB474115
23	8	21 November, 2003	URI	HRVi/Yamagata.JPN/47-4.03	4, 5	AB474116
24	1	16 December, 2003	Wheezy bronchiolitis	HRVi/Yamagata.JPN/51.03	5, 3	AB474117
25	2	17 December, 2003	URI	HRVi/Yamagata.JPN/51-2.03	10, 1	AB474118
26	7	21 January, 2004	URI	HRVi/Yamagata.JPN/4.04	5, 3	AB474119
27	13	30 March, 2004	URI	HRVi/Yamagata.JPN/14.04	2, 7	AB474120
28	3	26 April, 2004	URI	HRVi/Yamagata.JPN/18.04	2, 7	AB474121
29	7	26 April, 2004	URI	HRVi/Yamagata.JPN/18-2.04	5, 3	AB474122
30	2	7 May, 2004	URI	HRVi/Yamagata.JPN/19.04	10, 1	AB474123
31	10	2 June, 2004	URI	HRVi/Yamagata.JPN/23.04	2, 7	AB474124
32	0	28 July, 2004	URI	HRVi/Yamagata.JPN/31.04	6, 1	AB474125
33	0	16 August, 2004	URI	HRVi/Yamagata.JPN/34.04	4, 5	AB474126
34	1	14 September, 2004	URI	HRVi/Yamagata.JPN/38.04	4, 5	AB474127
35	2	2 November, 2004	URI	HRVi/Yamagata.JPN/45.04	6, 1	AB474128
36	4	20 April, 2005	URI	HRVi/Yamagata.JPN/16.05	6, 1	AB474129
37	1	12 May, 2005	URI	HRVi/Yamagata.JPN/19.05	6, 1	AB474130
38	2	16 May, 2005	URI	HRVi/Yamagata.JPN/20.05	9, 1	AB474131
39	3	24 May, 2005	URI	HRVi/Yamagata.JPN/21.05	9, 1	AB474132
40	6	13 October, 2005	URI	HRVi/Yamagata.JPN/41.05	11, 2	AB474133
41	11	18 October, 2005	URI	HRVi/Yamagata.JPN/42.05	11, 2	AB474134
42	4	28 February, 2006	URI	HRVi/Yamagata.JPN/9.06	5, 3	AB474135
43	10	18 March, 2006	URI	HRVi/Yamagata.JPN/11.06	10, 1	AB474136
44	9	24 April, 2006	URI	HRVi/Yamagata.JPN/17.06	5, 3	AB474137
45	1	1 May, 2006	URI	HRVi/Yamagata.JPN/18.06	10, 6	AB474138
46	3	19 May, 2006	URI	HRVi/Yamagata.JPN/20.06	2, 7	AB474139
47	0	27 May, 2006	Wheezy bronchiolitis	HRVi/Yamagata.JPN/21.06	2, 7	AB474140
48	1	26 May, 2006	URI	HRVi/Yamagata.JPN/21-2.06	5, 3	AB474141
49	8	31 May, 2006	URI	HRVi/Yamagata.JPN/22.06	5, 3	AB474142
50	0	6 June, 2006	URI	HRVi/Yamagata.JPN/23.06	4, 7	AB474143
51	1	6 June, 2006	Wheezy bronchiolitis	HRVi/Yamagata.JPN/23-2.06	2, 7	AB474144
52	1	21 June, 2006	URI	HRVi/Yamagata.JPN/25.06	7, 1	AB474145
53	1	21 June, 2006	URI	HRVi/Yamagata.JPN/25-2.06	8, 2	AB474146
54	2	21 July, 2006	URI	HRVi/Yamagata.JPN/29.06	2, 7	AB474148
55	0	1 December, 2006	URI	HRVi/Yamagata.JPN/48-2.06	8, 1	AB474150
56	1	30 March, 2007	URI	HRVi/Yamagata.JPN/13.07	7, 1	AB474151
57	1	23 May, 2007	URI	HRVi/Yamagata.JPN/21.07	4, 5	AB474152
58	3	2 June, 2007	URI	HRVi/Yamagata.JPN/22.07	1, 8	AB474153
59	3	6 June, 2007	URI	HRVi/Yamagata.JPN/23.07	10, 6	AB474154
60	1	27 June, 2007	URI	HRVi/Yamagata.JPN/26.07	1, 7	AB474155
61	6	22 June, 2007	URI	HRVi/Yamagata.JPN/25.07	1, 7	AB474156
62	2	22 June, 2007	URI	HRVi/Yamagata.JPN/25-2.07	1, 7	AB474157
63	2	3 July, 2007	URI	HRVi/Yamagata.JPN/27.07	4, 5	AB474158
64	10	13 July, 2007	URI	HRVi/Yamagata.JPN/28.07	1, 7	AB474159
65	5	21 August, 2007	URI	HRVi/Yamagata.JPN/34.07	5, 3	AB474160
66	8	25 September, 2007	URI	HRVi/Yamagata.JPN/39.07	9, 4	AB474161
67	0	29 September, 2007	URI	HRVi/Yamagata.JPN/35.07	6, 1	AB474163
68	1	3 October, 2007	URI	HRVi/Yamagata.JPN/40-2.07	5, 3	AB474164
69	2	9 October, 2007	URI	HRVi/Yamagata.JPN/41.07	4, 5	AB474165
70	1	15 October, 2007	URI	HRVi/Yamagata.JPN/42.07	4, 5	AB474166
71	2	18 October, 2007	URI	HRVi/Yamagata.JPN/42-2.07	7, 1	AB474167
72	0	24 October, 2007	URI	HRVi/Yamagata.JPN/43.07	11, 2	AB474168
73	1	1 November, 2007	URI	HRVi/Yamagata.JPN/44.07	6, 1	AB474169
74	0	6 November, 2007	URI	HRVi/Yamagata.JPN/45.07	7, 1	AB474170
75	1	6 November, 2007	URI	HRVi/Yamagata.JPN/45-2.07	11, 2	AB474171
76	2	22 November, 2007	URI	HRVi/Yamagata.JPN/47.07	10, 5	AB474172

* URI: upper respiratory infection.
** Left number: cluster numbers as phylogenetic tree based on the nucleotide sequences.
*** Right number: cluster numbers as phylogenetic tree based on the amino acid sequences.

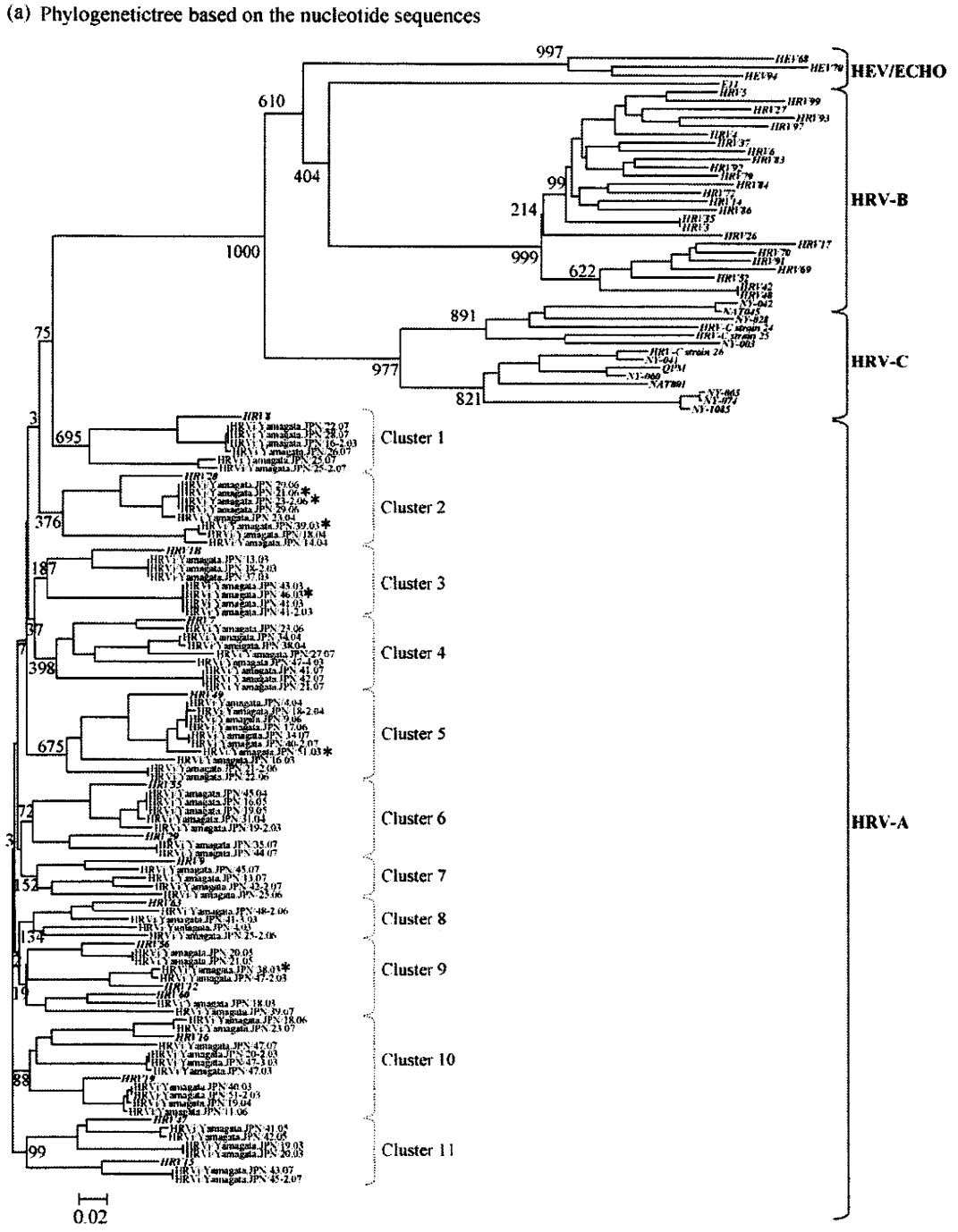


Fig. 1. Phylogenetic tree based on the *VP4/VP2* coding region of human rhinovirus species A (HRV-A), human rhinovirus species B (HRV-B), human rhinovirus species C (HRV-C), and human enterovirus species D (HEV-D). Echovirus 11(Echo-11), which belongs to the human enterovirus species B, was used as an outgroup. (a) Phylogenetic tree based on nucleotide sequences of the *VP4/VP2* coding region (390 nt) including the present strains (76 strains) and representative reference strains (16 strains). (b) Phylogenetic tree based on deduced amino acid sequences of the *VP4/VP2* coding region (130 aa) including the present strains and representative reference strains. Distance was calculated according to Kimura's 2-parameter method, and the tree was plotted with the neighbor-joining method. Numbers at each branch indicate the bootstrap values of the clusters supported by that branch. Reference strains are shown in bold type. (c and d) Phylogenetic trees based nucleotide sequences and amino acid sequences of the corresponding region for representative reference strains. GenBank accession numbers of references strains are as follows: HRV1B (GenBank accession no. D00239), HRV7 (AF343589), HRV8 (D00239), HRV9 (AF343605), HRV12 (AY016405), HRV15 (AF343630), HRV16 (L24917), HRV19 (AF343632) HRV28 (DQ473508), HRV29 (AF343615), HRV47 (FJ445133), HRV49 (AF343598), HRV55 (AF343621), HRV56 (FJ445140), HRV60 (AF343627), HRV63 (FJ445146), HRV3 (EF173422), HRV4 (AF343655), HRV5 (AF343651), HRV6 (DQ473486), HRV14 (K02121), HRV17 (AF343645), HRV26 (AF343653), HRV27 (AF343654), HRV35 (FJ445187), HRV37 (EF173423), HRV42 (FJ445130), HRV48 (DQ473488), HRV52 (FJ445188), HRV69 (FJ445151), HRV70 (AF343646), HRV72 (AF343650), HRV79 (FJ445155), HRV83 (AF343647), HRV84 (FJ445162), HRV86 (AF343648), HRV91 (FJ445168), HRV92 (FJ445169), HRV93 (EF173425), HRV97 (FJ445172), HRV99 (AF343652), HRV-C strain 24 (EF582385), HRV-C strain 25 (EF582386), HRV-C strain 26 (EF582387), QPM (EF186077), NY-003 (DQ875929), NY-028 (DQ875931), NY-041 (DQ875921), NY-042 (DQ875926), NY-060 (DQ875928), NY-063 (DQ875924), NY-074 (DQ875932), NY-1085 (DQ875925), NAT001 (FF077279), NAT045 (EF077280), HEV68 (EF107098), HEV70 (EV70CG), HEV94 (DQ916376), and Echo-11 (EU167522). *Strains from wheezy bronchiolitis.

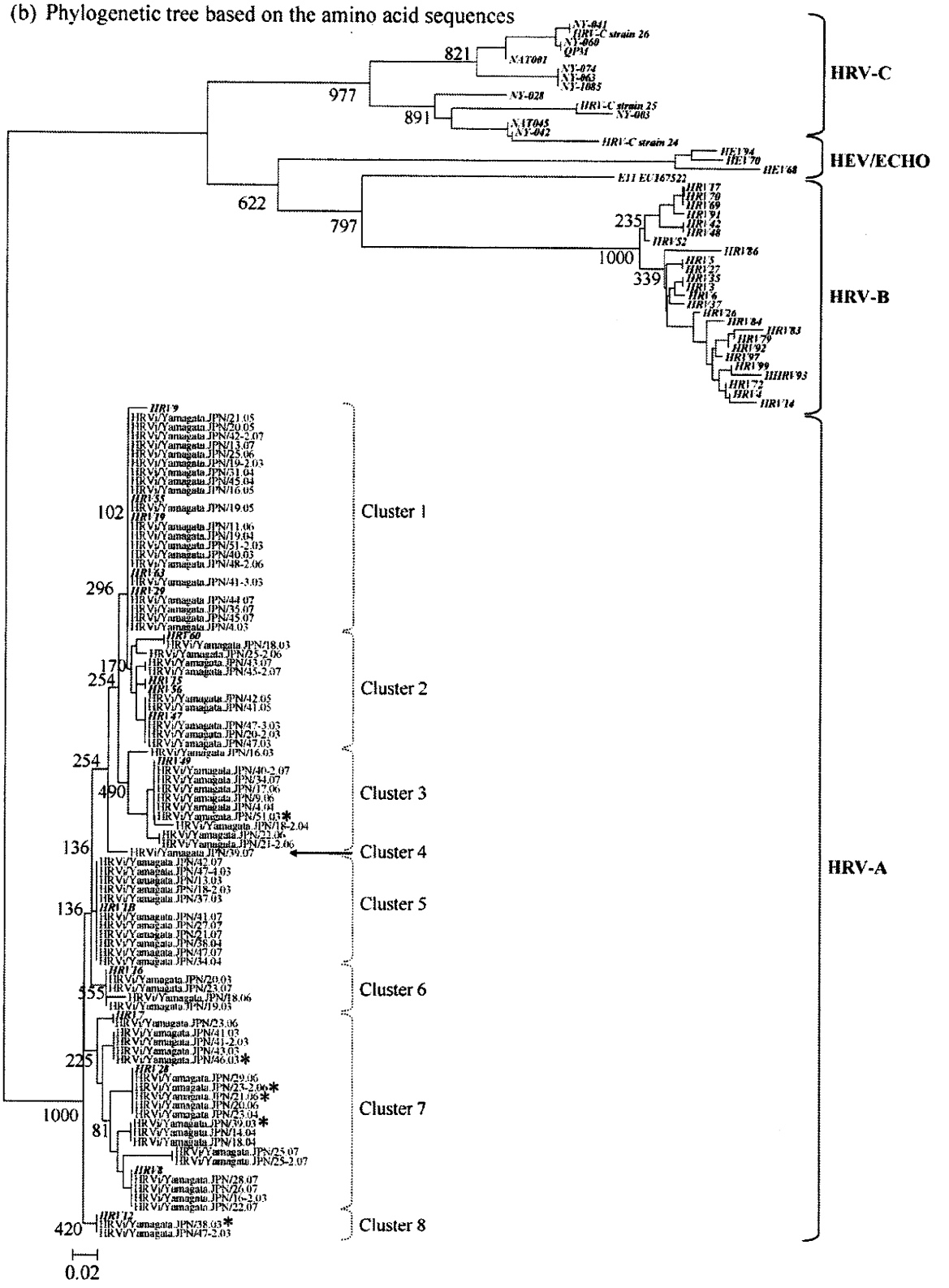
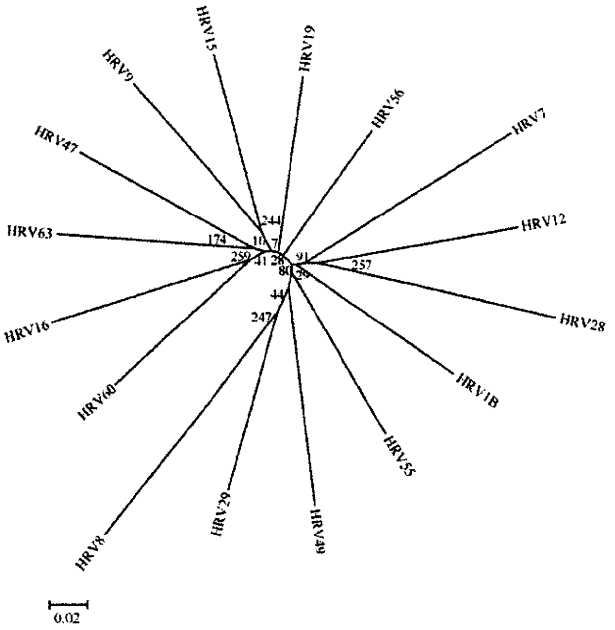


Fig. 1. (*Continued*)

(c) Phylogenetic tree based on the nucleotide sequences (reference strains)



(d) Phylogenetic tree based on the amino acid sequences (reference strains)

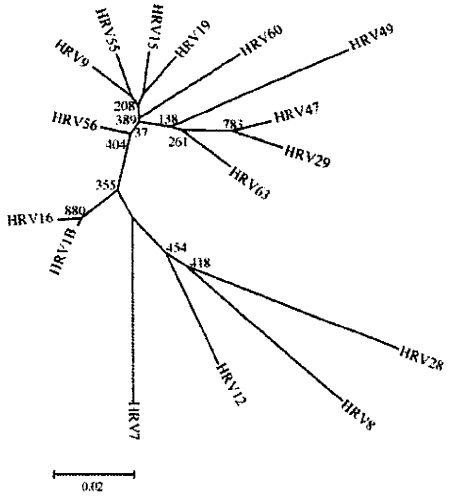


Fig. 1. (*Continued*).

3.2. Distribution of pairwise distances of interspecies of HRV-A based on the nucleotide and amino acid sequences

We calculated interspecies distances of HRV-A by the distribution of pairwise distances (Figs. 2a–k and 3a–i). Among the present and reference strains, the interspecies distances based on the nucleotide and amino acid sequences were 0.235 ± 0.048 and 0.076 ± 0.033 (mean ± SD), respectively (Figs. 2a, 3a). The intercluster distances based on the nucleotide sequences were as follows: Cluster 1, 0.136 ± 0.101 (mean ± SD, Fig. 2b); Cluster 2, 0.115 ± 0.086 (c); Cluster 3, 0.152 ± 0.115 (d); Cluster 4, 0.142 ± 0.062 (e); Cluster 5, 0.085 ± 0.058 (f); Cluster 6, 0.130 ± 0.093 (g); Cluster 7, 0.159 ± 0.053 (h); Cluster 8, 0.167 ± 0.053 (i); Cluster 9, 0.184 ± 0.064 (j); Cluster 10, 0.169 ± 0.072 (k) and Cluster 11, 0.153 ± 0.072 (l). Next, the intercluster distances based on the amino acid sequences were as follows: Cluster 1, 0.031 ± 0.020 (mean ± SD, Fig. 3b); Cluster 2, 0.040 ± 0.016 (c); Cluster 3, 0.012 ± 0.012 (d); Cluster 5, 0.030 ± 0.025 (e); Cluster 6, 0.027 ± 0.020 (f); Cluster 7, 0.079 ± 0.041 (g); and Cluster 8, 0 (h). The longest pairwise intercluster distances based on the nucleotide and amino acid sequences were Cluster 9 and Cluster 7, respectively, whereas the shortest pairwise distances were Cluster 5 and Cluster 8, respectively.

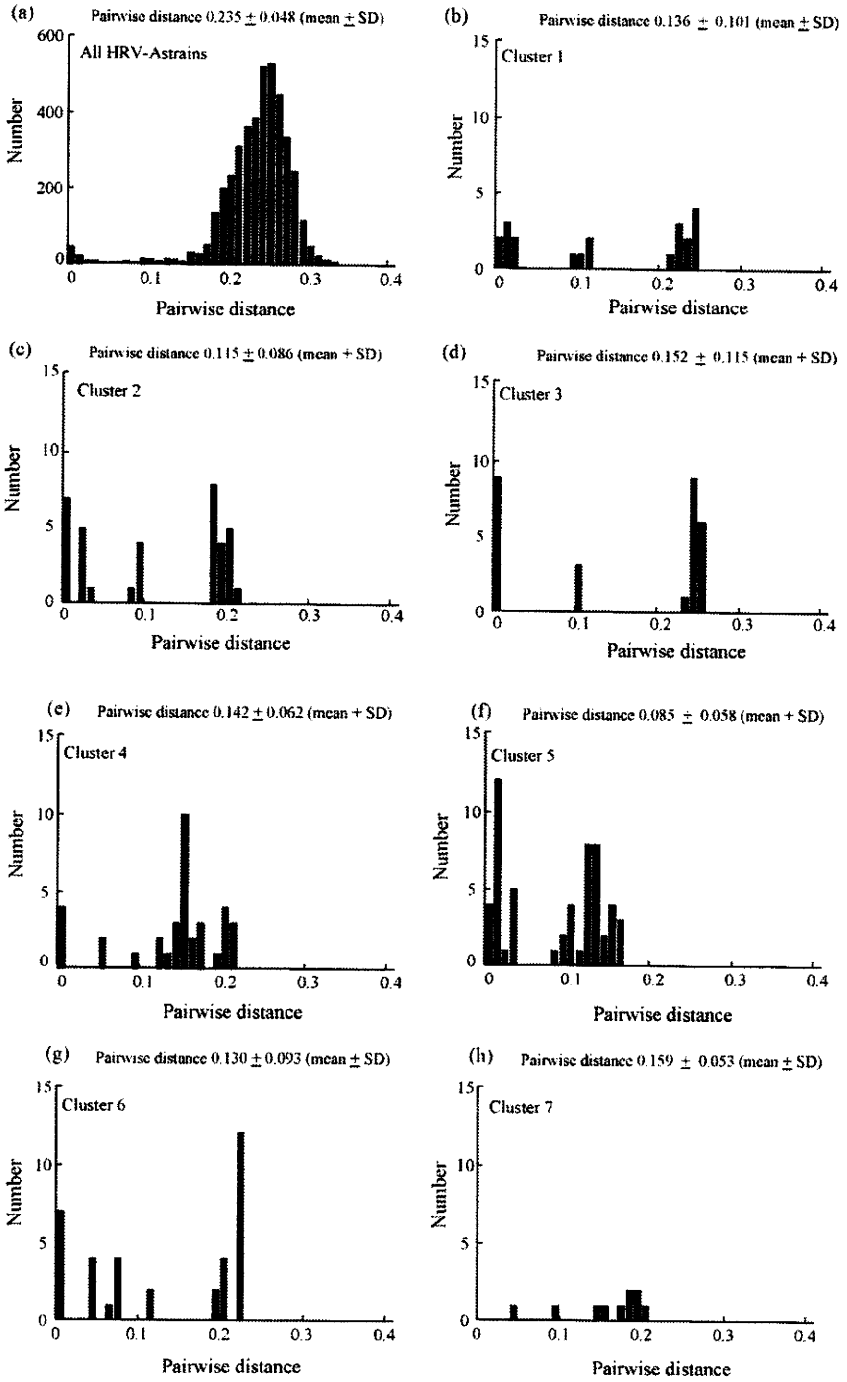


Fig. 2. Distributions of pairwise interspecies distances and pairwise distances for each intercluster for HRV-A based on nucleotide sequences of *VP4/VP2* coding region (390 nt). (a) Distribution of pairwise interspecies distances based on nucleotide sequences of the *VP4/VP2* coding region. (b–l) Distributions of pairwise distances for each intercluster (Clusters 1–11).

4. Discussion

To ascertain the address molecular epidemiology of domestic HRV-A infection, we performed phylogenetic and cluster analysis of the *VP4/VP2* coding region of prevalent strains (76 isolates) isolated from children with ARI in Yamagata prefecture, Japan during the period 2003–2007. Phylogenetic analysis based on the nucleotide and deduced amino acid sequences showed that the present HRV-A strains were clearly classified into 11 and 8 clusters, respectively. These viruses showed more than 30% of nucleotide divergence of the *VP4/VP2* cording region. The findings suggest that HRV-A with a wide genetic divergence was associated with URI and wheezy bronchiolitis during the investigation period in the study area.

Matsumoto et al. (1991) reported that approximately 32% of Japanese patients with ARI had associated HRV, although viruses were confirmed by a physicochemical method. Another study

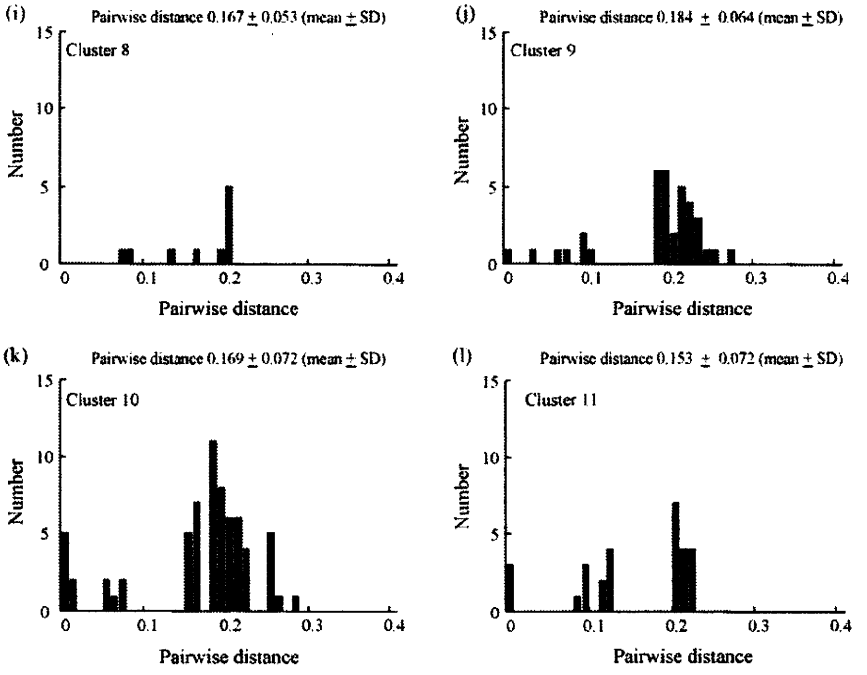


Fig. 2. (*Continued*).

found HRV in hospitalized infants with bronchiolitis and wheezing (Jartti et al., 2009). Calvo et al. (2007) reported that 25% of hospitalized infants with ARI had HRV infection as confirmed by multiplex PCR. In addition, HRV infections are frequently found in patients hospitalized with pneumonia (Cheuk et al., 2007). Savolainen et al. (2002a) showed that some serotypes of HRV, including HRV45, 78, and 81, were frequently associated with ARI in Finland. In American children with wheezing, various serotypes of HRV, including HRV30, 44, and 49, were found (Khetsuriani et al., 2008). In the present study, approximately 90% of patients with ARI had URI, and approximately 10% had wheezy bronchiolitis. Recent studies have shown that HRV can be classified into several species (Savolainen et al., 2002a,b). Savolainen et al. (2002b) reported that 61 isolates from patients with ARI could be classified into genetic groups 1 and 2, and these genetic groups could be further categorized into 12 clusters. Relations between genetic and serologic HRV type have been studied (Savolainen et al., 2002a). Most prototype HRV strains (isolates) have been classified into 2 species (HRV-A and -B). Of them, viruses belonging to species HRV-A comprise the major agent associated with ARI (Savolainen et al., 2002a). HRV-A can be subclassified into various clusters (Savolainen et al., 2002a). For example, McErlean reported that frequently detected HRV strains can be classified into 3 genotypes (Types A1, A2, and B) (McErlean et al., 2007). However, the detailed molecular epidemiology of HRV is not known. Moreover, to the best of our knowledge, there have been few molecular epidemiologic studies of HRV-A in Asian countries including Japan. Therefore, we performed a detailed phylogenetic and cluster analysis of HRV-A of patients with ARI, such as upper respiratory infections and wheezy bronchiolitis, in Yamagata prefecture, Japan. We found that prevalent HRV-A strains isolated from Japanese patients with ARI showed a wide nucleotide divergence. The data suggested that HRV-A strains belonging to some clusters were associated with wheezy bronchiolitis. However, we do not know whether these viruses can easily cause wheezy bronchiolitis because only 6 cases of wheezy bronchiolitis caused by HRV-A were examined in the present study. Recent evidence suggests that some genotypes of respiratory viruses, such as HRV, respiratory syncytial virus (RSV), and parainfluenza viruses, may be linked to virus-induced asthma (Jartti et al., 2004, 2007; Martinello et al., 2002). For example, a specific genotype (GA 3) of RSV may be associated with significantly greater severity of illness (Martinello et al., 2002). However, another report showed no association between severity of illness and RSV subgroups, and the severity was instead associated with the amount of RSV in nasopharyngial aspirates (Campanini et al., 2007; Sato et al., 2005). Thus, the association between a specific virus type and the severity of RSV infections, including bronchiolitis, has not been precisely addressed. In addition, Johnston (2007) reported that HRV infection is strongly related to virus-induced asthma or exacerbation of asthma. As possible reason for this association with HRV-induced asthma is insufficient production of the cytokine interferon-β or λ; however, the molecular mechanisms underlying this are not fully understood as yet (Johnston, 2007). Additional studies with large numbers of patients with HRV-induced airway hyperresponsiveness, including asthma, would be beneficial.

The *VP4/VP2* coding region encodes interior viral capsid. Genetic clustering on the basis of the *VP4/VP2* sequences was reported to be an effective method for the identification of many types of HRV isolates (Savolainen et al., 2002a). To better perform phylogenetic and cluster analysis of many types of HRV-A in the present study, we selected the *VP4/VP2* coding region, which resulted in the construction of a clearly distinguishable phylogenetic tree (Savolainen et al., 2002a; Kiang et al., 2007). Thus, the use of this coding region for phylogenetic analysis of HRV may be suitable for the clustering of many field isolates of HRV, consistent with earlier reports (Savolainen et al., 2002a,b).

We were able to collect clinical specimens from only a relatively small number of pediatric patients with ARI in a limited area (Yamagata prefecture), covering only a small number of HRV-A strains from children who attended a pediatric clinic. In addition, no seasonal variation of HRV was found in the present data, but it may be important to address seasonal variation regarding HRV epidemiology as a future research topic. To address the