pluripotency, J Biol Chem, 286: 11593-11603 2011

## 2. 学会発表等

(1) K Miyakawa, M Nishi, N Yamamoto, A Ryo: The Tumor Suppressor APC Regulates HIV-1 Assembly and Release. IUMS 2011 International Congress of Virology, Sapporo, Sep 11-16, 2011, Sapporo, Sep 11-16, 2011.

(2) Matsunaga S, Furukawa A, Kojima Y, Morishita R, Sawasaki T, Takaori-kondo A, Sugiura W, Katahira M, Ryo A: An in vitro cleavage assay system for XMRV protease by wheat-germ cell free protein production. IUMS 2011 International Congress of Virology, Sapporo, Sep 11-16, 2011.

(3) Kanuma T, Kudoh A, Jounai N, Takeshita F, Sawasaki T, Ryo A: Immune evasion by HIV-1protease-mediated cleavage of TBK1. IUMS 2011 International Congress of Virology, Sapporo, Sep 11-16, 2011.

(4) Miyakawa K, Sawasaki T, Matsunaga S, Kimura H, Yamamoto N, Guatelli J, Ryo A: Identification of SCYL2 as an interferon- inducible antagonist of HIV-1 Vpu. 6th German- Japanese HIV Symposium, Ruhr-Universität Bochum, German, Nov 21-22,2011.

(5) Matsunaga S, Sawasaki T, Ode H, Morishita R, Sugiura S, Sato H, Ryo A: An in vitro cleavage assay system for retrovirus protease by wheat germ cell-free protein production. 6th German-Japanese HIV Symposium, Ruhr-Universität Bochum, German, Nov 21-22, 2011.

(6) 松永智子, 澤崎達也, 小島良績, 森下 了, 佐藤裕徳, 大出裕高, 杉浦 亙, 梁 明秀: コムギ無細胞タンパク質合成系を用いた Xenotropic murine leukemia virus-related virus (XMRV) プロテアーゼの解析. 日本プロテオーム学会, 2011年7月28～29日, 新潟.

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分担研究報告書

# RS ウイルス感染ヒト胎児肺線維芽細胞によって産生が誘導されるサイトカインとシグナル伝達経路との相互関係に関する研究

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**研究要旨**

幼少期における呼吸器ウイルス感染は、喘息の発症と増悪に密接に関連していることが強く示唆されている。また、呼吸器に存在する線維芽細胞は、喘息患者に生じる気道リモデリングに重要な役割を果たすと考えられている。本研究では、RS ウイルス（RSV）感染ヒト肺線維芽細胞(MRC-5 細胞)が産生するサイトカインのプロファイリングを行うとともにサイトカイン産生に重要なシグナル伝達機構解明を目的とした研究を行った。さらに、炎症性疾患に対する治療に広く用いられているステロイド（Fluticasone propionate）の抗炎症効果を *in vitro* で検証した。その結果、RSV 感染 MRC-5 細胞は、種々の炎症性サイトカインや気道リモデリングに関連するサイトカインを多量に産生することが明らかになった。これらのうち、特に Th2 サイトカインの産生は Fluticasone propionate により有意に抑制された。また、このサイトカイン産生には Akt、p38MAPK、ERK1/2 および IκBα のリン酸化が密接に関与することも明らかになった。以上のことから、RSV の感染によって引き起こされる大量のサイトカイン産生は、感染による過剰な炎症とアレルギー性炎症および気道リモデリングに関与することが示唆された。

## A. 研究目的

RS ウイルス(Respiratory Syncytial Virus, RSV)は、乳児のウイルス性気管支炎や肺炎の原因となる主要な呼吸器ウイルスであり、他のウイルス（インフルエンザウイルス）と同様に再感染を引き起こすことが知られている(1)。

RSV を含む一部の呼吸器ウイルスは、喘鳴の発症や増悪の病態に関与しており、さらに喘息の発症・増悪(virus-induced asthma)の誘導に深く関与することが報告されている (2, 3)。この病態には、ウイルス感染によって、感染細胞から産生が誘導されるさまざまなサイトカインが深く関与することも示唆されている(4, 5)。また、これらのサイトカイン産生と気道リモデリングには、肺組織に多く含まれる線維芽細胞が深く関与していることが推察される (6)。

また、強い抗炎症効果を有するコルチコステロイドは、喘息などのアレルギー性疾患を含め、臨床において広汎に用いられている。その作用機序について、多くの知見が集積し

つつあるが(7)、ウイルス感染によって引き起こされる過剰な炎症反応や virus-induced asthma の本剤の抑制機構については、いまだ解明に至っていないと思われる。

そこで、本研究においては、RSV 感染胎児肺線維芽細胞（MRC-5 細胞）によって惹起されるサイトカイン産生ならびにサイトカイン産生に関与するシグナル伝達機構解明、さらに、これらサイトカイン産生系へのステロイド抑制機構の解明を目的とした以下の内容の研究を行った。

## B. 研究方法

1. ウイルス

RSV (Long strain, VR-26)を HEp-2 細胞にて培養・増殖した後、ショ糖密度勾配法を用いて回収したウイルス液を試料として用いた。

2. サイトカインおよびリン酸化シグナルタンパクの測定

ヒト胎児肺線維芽細胞(MRC-5, ATCC; CCL-171)を、常法により培養した後、マイクロプレートに播種し、MRC-5 細胞に 1 MOI の RSV を感染させ、24 時間後の培養上清および細胞を回収した。サイトカイン産生とシグナルタンパク産生との関連性について検討するため、培養上清中に含まれる 29 種類のサイトカインの解析および RSV 感染 MRC-5 細胞のリン酸化シグナル関連タンパク質について解析を行った。

## C. 研究結果

感染 24 時間後では、炎症性サイトカインの IL-1β、 IL-6、TNF-α、抗炎症性サイトカインの IL-1ra、Th1 サイトカインの IFN-γ および IL-2、IFN-λ1a、Th2 サイトカインの IL-4、IL-5、IL-10、および IL-13、造血因子の G-CSF および GM-CSF、好中球遊走サイトカインの IL-8 および IP-10、 好酸球遊走サイトカインの eotaxin,および RANTES 等、多くのサイトカインが有意に産生された(図 1)。これらのサイトカイン産生はステロイド剤である Fluticasone propionate により有意に抑制された(図 1)。また、RSV 感染により、Akt、p38MAP、ERK1/2、および IκBα のリン酸化が有意に増強した(図 2)。これらのシグナル蛋白のリン酸化は Fluticasone propionate により有意に抑制されたが、IκBα のリン酸化は阻害されなかった(図 2)。

## D. 考察

本研究により、RSV 感染によって、ヒト胎児肺線維芽細胞が Akt、p38MAPK、ERK1/2 および IκBα のシグナル経路を介して多くのサイトカインを産生・放出することが明らかになった。これらの多量のサイトカイン産生とサイトカインバランスの不均衡が、部分的にではあるが喘息の病態生理や気道リモデリングに関与していることが示唆された。

また、今回の結果から、RSV は、肺の気道上皮細胞のみならず線維芽細胞にも感染し、種々のサイトカイン産生を亢進することも明らかになった。このことは、RSV 感染によって生ずる重症化、特にサイトカインストームへの関与も示唆する。さらに、これらのシグナル伝達経路として Akt、p38MAPK、ERK1/2 および IκBα のリン酸化が特異的に関与していることも推察された。多くのサイトカインのシグナル蛋白とされる NF-κB は、阻害蛋白である IκBα により核内移行を抑制されているが、ウイルス感染などの刺激により IκBα のリン酸化が起こり、IκBα から遊離した NF-κB の核内移行によりサイトカイン産生シグナルが核に伝達される(8)。ステロイドが IκBαmRNA を誘導し IκBα を増加させることにより NF-κB の核内移行を抑制しているとの報告もあり(9, 10)、本研究結果もこれに準ず

るものと考えられる。このようにステロイドによるシグナル伝達機構の抑制がサイトカイン産生の抑制に関与することも示唆され、RSV感染により引き起こされる喘息の病態緩和につながると考えられた。

結論として、RSV感染による肺線維芽細胞の炎症性サイトカインを中心としたサイトカインの異常産生による生体反応機構は、単に呼吸器感染症として、急性の気道炎症を惹起するだけでなく、喘息の発症や増悪および気道リモデリング等の重要な機構になっていると考えられる。

## E. 結論

本研究により、RSV感染によりヒト肺線維芽細胞から、種々のサイトカイン産生が誘導されることが明らかになった。この産生機構には、Akt、p38MAPK、ERK1/2 および IκBα のリン酸化が重要であることが判明した。また、産生された多量のサイトカインが種々の病態、サイトカインストーム、喘息の発症増悪および気道リモデリングに関与することも推察された。

## F. 参考文献


1. Domachowske, J. B., Rosenberg, H. F. Respiratory syncytial virus infection; Immune response, immunopathogenesis, and treatment. Clin Microbiol Rev 1999: 12: 298-309.

2. McIntosch, K., Ellis, E. F., Hoffman, L. S., Lybass, T. G., Eller, J. J., Fulqiniti, V. A. Association of viral and bacterial respiratory infections with exacerbations of wheezing in young asthmatic children. J Pediatr 1973; 82: 578-590.

3. Johnston, S. L. Overview of virus-induced airway disease. Proc. Am. Thorac. Soc. 2004; 2: 150–156.

4. Kotaniemi-Syrjänen, A., Vainionpää, R., Reijonen, T. M., Waris, M., Korhonen, K., and Korppi, M. Rhinovirus-induced wheezing in infancy--the first sign of childhood asthma? J. Allergy Clin. Immunol. 2003; 111:66–71.

5. Martinez, F. D. Respiratory syncytial virus bronchiolitis and the pathogenesis of childhood asthma. Pediatr. Infect. Dis. J. 2003;

6. Barnes, P. J. The cytokine network in asthma and chronic obstructive pulmonary disease. J. Clin. Invest. 2008; 118: 3546–3556.

7.Barrett, N. A., and Austen, K. F. Innate cells and T helper 2 cell immunity in airway inflammation. Immunity; 2009; 31: 425–437.

8. Puxeddu, I., Bader, R., Piliponsky, A. M., Reich, R., Levi-Schaffer, F., and Berkman, N. The CC chemokine eotaxin/CCL11 has a selective profibrogenic effect on human lung fibroblasts. J. Allergy Clin. Immunol. 2006; 117: 103–110.

9. Barnes, P. J. How corticosteroids control inflammation: Quintiles prize lecture 2005. Br J Pharmacol 2006; 148: 245-254.

10. Tak, P. P., Firestein, G. S. NF-κB: a key role in inflammatory diseases. J Clin Invest 2001; 107: 7-11.

11. Auphan, N., Didonato, J. A., Rosette, C., Helmberg, A., Karin, M. Immunosuppression by glucocorticoids; inhibition of NF-B activity through induction of IB synthesis. Science 1995; 270: 286-290.

12. Scheinman, R. I., Cogswell, P. C., Lofquist, A. K., Baldwin, A. S. Jr. Role of transcriptional activation of IB in mediation of immunosuppression by glucocorticoids. Science 1995; 270: 283-286.


## G. 研究発表

### 1. 論文発表

1) Fujitsuka A, Tsukagoshi H, Arakawa M, Goto-Sugai K, Ryo A, Okayama Y, Mizuta K, Nishina A, Yoshizumi M, Kaburagi Y, Noda M, Tashiro M, Okabe N, Mori M, Yokota S, Kimura H . A molecular epidemiological study of respiratory viruses detected in Japanese children with acute wheezing illness. BMC Infect Dis. 2011;11:168.

2) Tanaka T, Yokoi H, Kobayashi K, Iwanade H, Noguchi Y, Mitsui Y, Okamoto A, Saitoh M, Noda M, Takeda M, Okabe N, Kimura H. First detection of measles virus genotype g3 in a Japanese woman: an imported case. Jpn J Infect Dis. 2011;64:262-3.

3) Itagaki T, Abiko C, Aoki Y, Ikeda T, Mizuta K, Noda M, Kimura H, Matsuzaki Y. Saffold cardiovirus infection in children associated with respiratory disease and its similarity to coxsackievirus infection. Pediatr Infect Dis J. 2011;30:680-3.

4) Nakamura M, Taira K, Tsukagoshi H, Itokazu K, Nidaira M, Okano S, Kudaka J, Noda M, Takeda M, Kimura H. Detection of various respiratory viruses in patients with influenza-like illness before and after emergence of the 2009 pandemic H1N1 influenza virus in Okinawa. Jpn J Infect Dis. 2011;64:87-9.

5) Omura T, Iizuka S, Tabara K, Tsukagoshi H, Mizuta K, Matsuda S, Noda M, Kimura H. Detection of human metapneumovirus genomes during an outbreak of bronchitis and pneumonia in a geriatric care home in Shimane, Japan, in autumn 2009. Jpn J Infect Dis. 2011;64:85-7.

**H. 知的財産の出願・登録状況**

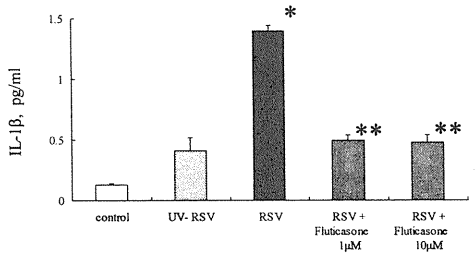
なし

1 MOI / 24hr

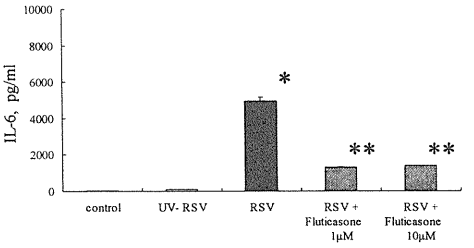
* $P < 0.05$; control vs RSV 1MOI
* * $P < 0.05$; RSV 1MOI vs Fluticasone

## Proinflammatory cytokines

### IL-1β




### IL-6

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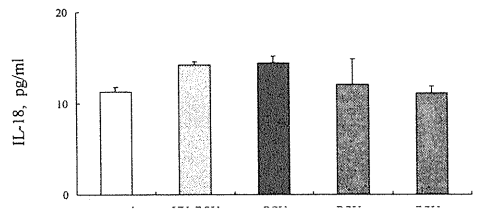
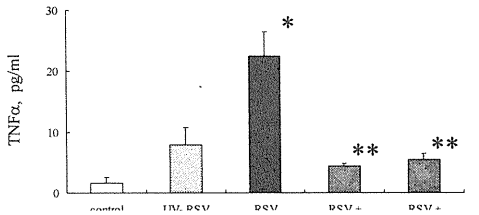
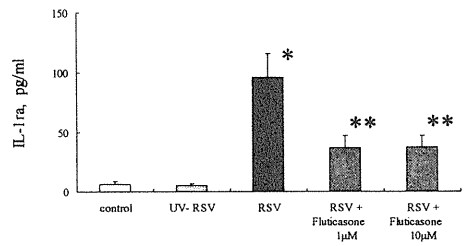



### TNFα

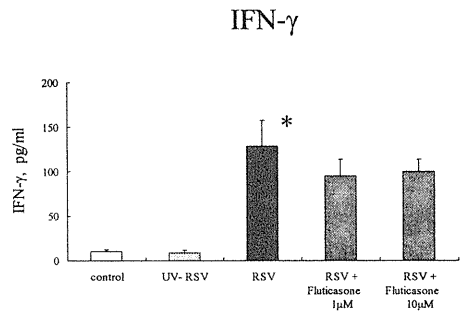



## Anti-iflammatory cytokine

### IL-1ra

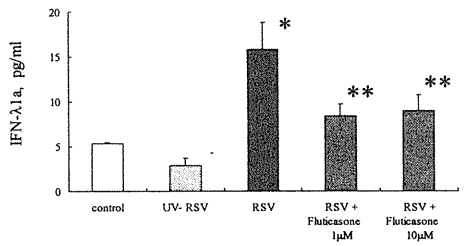



### IFN-γ




## Th1 cytokines

### IFN-λ1a




### IL-2

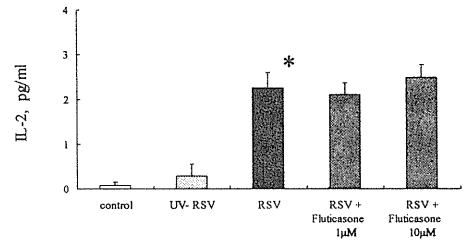
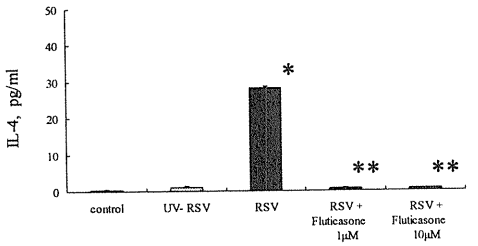


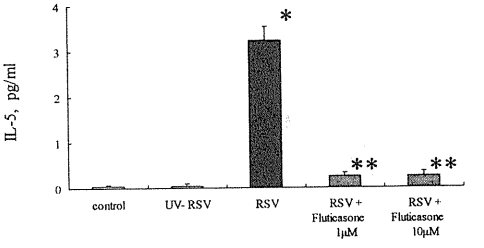

図１　RSV 感染肺線維芽細胞からのサイトカイン産生

Th2 cytokines

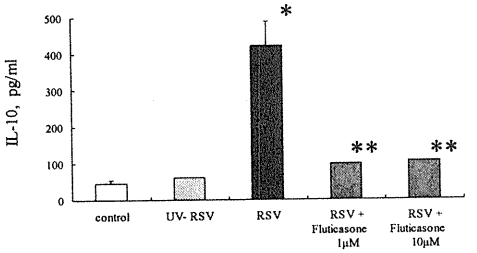
IL-4



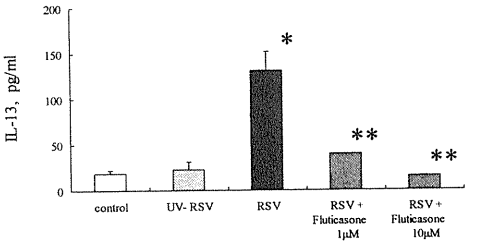

IL-5




IL-10

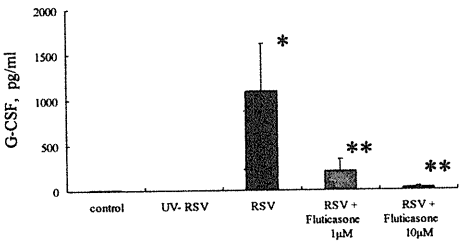



IL-13

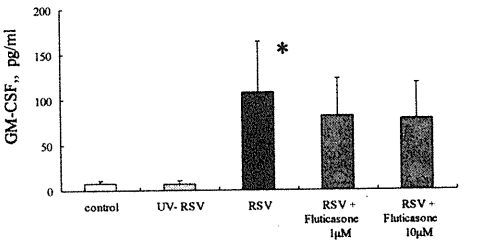



Granulopoiesis-inducing cytokines

G-CSF

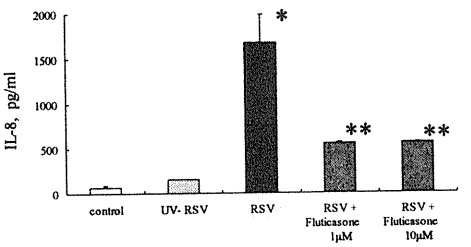



GM-CSF




Neutrophil recruitment-inducing cytokines

IL-8




IP-10

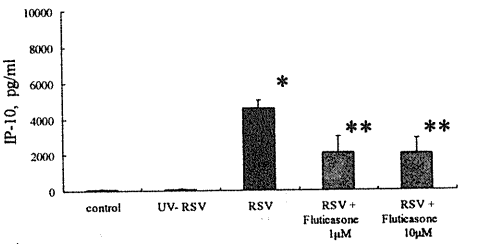



図1　RSV感染肺線維芽細胞からのサイトカイン産生（続き）

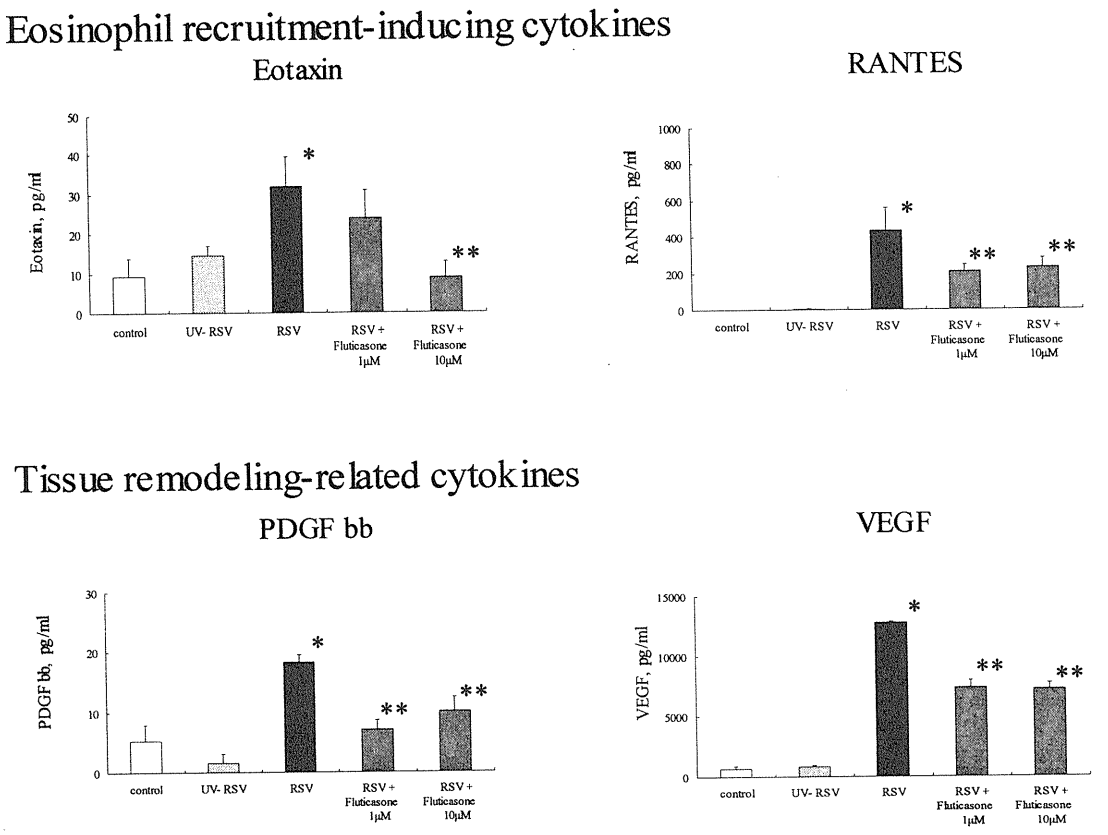



図1　RSV 感染肺線維芽細胞からのサイトカイン産生（続き）

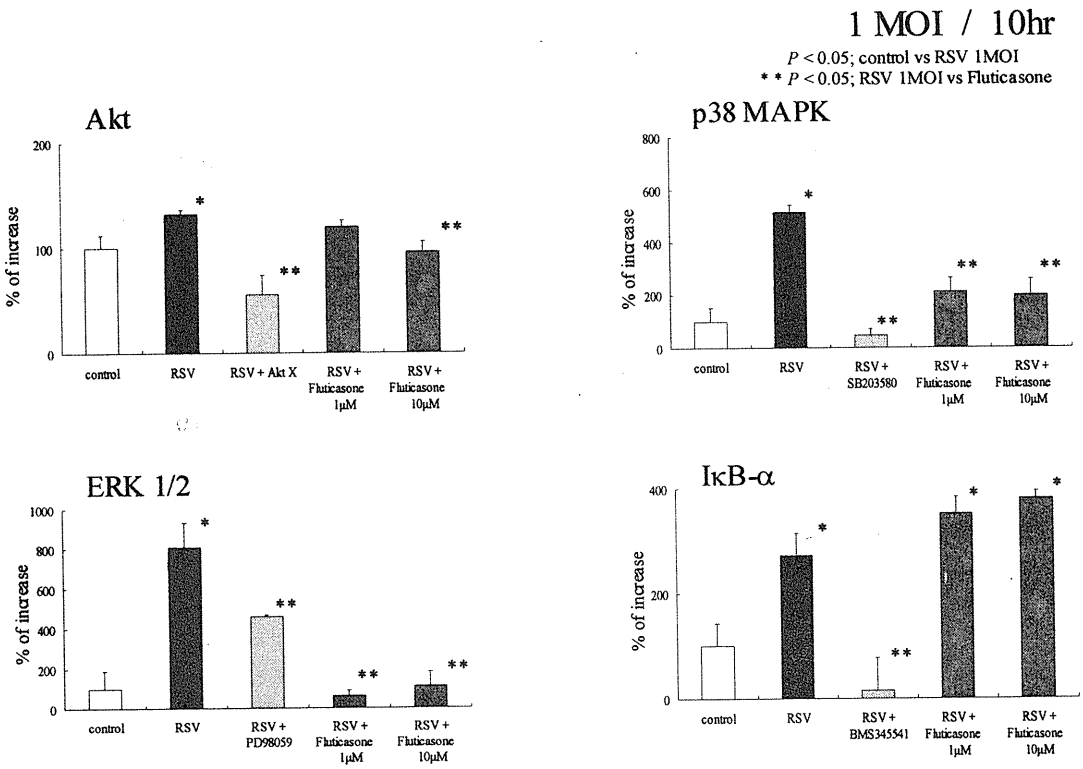



図2　RSV 感染肺線維芽細胞からのリン酸化シグナルタンパク産生

研究成果の刊行に関する一覧表


1. Ikeda T, Mizuta K, Abiko C, Aoki Y, Itagaki T, Katsushima F, Katsushima Y, Matsuzaki Y, Fuji N, Imamura T, Oshitani H, Noda M, Kimura H, Ahiko T. Acute respiratory infections due to enterovirus 68, in Yamagata, Japan between 2005 and 2010. Microbiol Immunol. in press.
2. Arakawa M, Okamoto-Nakagawa R, Toda S, Tsukagoshi H, Kobayashi M, Ryo A, Mizuta K, Hasegawa S, Hirano R, Wakiguchi H, Kudo K, Tanaka R, Morita Y, Noda M, Kozawa K, Ichiyama T, Shirabe K, Kimura H. Molecular epidemiological study of human rhinovirus species ABCs from patients with acute respiratory illnesses in Japan. J Med Microbiol. 61(Pt 3):410-9, 2012.
3. Nishina A, Kimura H, Kozawa K, Sommen G, Nakamura T, Heimgartner H, Koketsu M, Furukawa S. A superoxide anion-scavenger, 1,3- selenazolidin-4-one suppresses serumdeprivation-induced apoptosis in PC12 cells byactivating MAP kinase. Toxicol Appl Pharmacol.2011; in press.
4. Mizuta K, Saitoh M, Kobayashi M, Tsukagoshi H, Aoki Y, Ikeda T, Abiko C, Katsushima N, Itagaki T, Noda M, Kozawa K, Ahiko T, Kimura H. Detailed genetic analysis of hemagglutinin-neuraminidase glyco-protein gene in human parainfluenza virus type 1isolates from patients with acute respiratory infectionbetween 2002 and 2009 in Yamagata prefecture, Japan. Virol J; in press.
5. Sakano C, Morita Y, Goto K, Yokota Y, Annaka H, Fujita M, Kobatake S, Ishioka T, Hoshino T, Boonmar S, Pulsrikarn C, Nishina A, Kozawa K, Yamamoto S, Kimura H. Prevalence and genotype of Salmonella Choleraesuis in Gunma Prefecture, Japan. Thai J Vet Med. 41(3):321-326, 2011.
6. Itagaki T, Abiko C, Aoki Y, Ikeda T, Mizuta K, Noda M, Kimura H, Matsuzaki Y. Saffold cardiovirus infection in children associated with respiratory disease and the similarity to coxsackievirus infection. Pediatr Infect Dis J. 30(8):680-683, 2011.
7. Tsukagoshi H, Mizuta K, Abiko C, Itagaki T, Yoshizumi M, Kobayashi M, Kuroda M, Kozawa K, Noda M, Ryo A, Kimura H. The impact of Saffold cardiovirus in patients with acute respiratory infections in Yamagata, Japan. Scand J Infect Dis. 2011; 43(8):669-71.
8. Fujitsuka A, Tsukagoshi H, Arakawa M, Goto-Sugai K, Ryo A, Okayama Y, Mizuta K, Nishina A, Yoshizumi M, Kaburagi Y, Noda M, Tashiro M, Okabe N, Mori M, Yokota S, Kimura H. A molecular epidemiological study of respiratory viruses detected in Japanese children with acute wheezing illness. BMC Infect Dis. 11:168, 2011.
9. Nishi M, Akutsu M, Masui S, Kondo A, Nagashima Y, Kimura H, Perrem K, Shigeri Y, Toyoda M, Okayama A, Hirano H, Umezawa A, Yamamoto N, Sam W. Lee SW, Ryo A. A distinct role for Pin1 in the induction and maintenance of pluripotency. J Biol Chem. 286(13):11593-11603, 2011.
10. Nishina A, Kimura H, Kozawa K, Sommen G, Favero F, Heimgartner H, Koketsu M, Furukawa S. 3,3-(2,6-Dimethylphenyl)-2-Selenoxo-1,3-Thiazolidin-4-One suppresses hydrogen peroxide-inducedcytotoxicity on PC12 cells via activation of MAPK.Int J Toxicol. 2011; in press.
11. Ishioka T, Kimura H, Kita H, Obuchi M, Hoshino H, Noda M, Nishina A, Kozawa K, Kato M. Effects of respiratory syncytial virus infection and major basic protein derived from eosinophils in pulmonary alveolar epithelial cells (A549). Cell Biol Int. .2011; 35(5):467-74.

12. Nakamura M, Taira K, Tsukagoshi H, Itokazu K, Nidaira M, Okano S, Kudaka J, Noda M, Takeda M, Kimura H. Detection of various respiratory viruses in patients with influenza-like illness before and after emergence of the 2009 Pandemic H1N1 influenza virus in Okinawa. Jpn J Infect Dis. 64(1):87-89, 2011.
13. Hasegawa S, Hirano R, Okamoto-Nakagawa R,Ichiyama T, Shirabe K, . Enterovirus 68 infection in children with asthma attacks: Virus-induced asthma in Japanese children Allergy 2011; 66(12) 1618-1620
14. Tanaka T, Yokoi H, Kobayashi K, Iwanade H, Noguchi Y, Mitsui Y, Okamoto A, Saitoh M, Noda M, Takeda M, Okabe N, Kimura H. First detection of measles virus genotype G3 in a Japanese woman:An imported case. Jpn J Infect Dis. 64(3):262-263, 2011.
15. Omura T, Iizuka S, Tabara K, Tsukagoshi H, Mizuta K, Matsuda S, Noda M, Kimura H. Detection of human metapneumovirus genomes during an outbreak of bronchitis and pneumonia in a geriatric care home in Shimane, Japan, in autumn 2009. Jpn J Infect Dis. 2011;64(1):85-7.

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# Molecular epidemiological study of human rhinovirus species A, B and C from patients with acute respiratory illnesses in Japan

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Recent studies suggest that human rhinovirus species A, B and C (HRV-ABCs) may be associated with both the common cold and severe acute respiratory illnesses (ARIs) such as bronchiolitis, wheezy bronchiolitis and pneumonia. However, the state and molecular epidemiology of these viruses in Japan is not fully understood. This study detected the genomes of HRV-ABCs from Japanese patients (92 cases, 0–36 years old, mean ± SD 3.5 ± 5.0 years) with various ARIs including upper respiratory infection, bronchiolitis, wheezy bronchiolitis, croup and pneumonia between January and December 2010. HRV-ABCs were provisionally type assigned from the pairwise distances among the strains. On phylogenetic trees based on the nucleotide sequences of the *VP4/VP2* coding region, HRV-A, -B and -C were provisionally assigned to 14, 2 and 12 types, respectively. The present HRV-A and -C strains had a wide genetic diversity (>30 % divergence). The interspecies distances were 0.230 ± 0.063 (mean ± SD, HRV-A), 0.218 ± 0.048 (HRV-B) and 0.281 ± 0.105 (HRV-C), based on nucleotide sequences, and 0.075 ± 0.036 (HRV-A), 0.049 ± 0.022 (HRV-B) and 0.141 ± 0.064 (HRV-C) at the deduced amino acid level. Furthermore, HRV-A and -C were the predominant species and were detected throughout the seasons. The results suggested that HRV-A and -C strains have a wide genetic divergence and are associated with various ARIs in Japan.

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Abbreviations: ARI, acute respiratory illness; HRV-ABCs, human rhinovirus species A, B and C; NPS, nasopharyngeal swab; URI, upper respiratory infection.

The GenBank/EMBL/DDBJ accession numbers for the HRV sequences determined in this study are AB628096–AB628187.

## INTRODUCTION

Human rhinoviruses (HRVs) are a group of positive-sense ssRNA viruses belonging to the genus *Enterovirus* in the family *Picornaviridae*. Previous reports have suggested that HRVs are responsible for various acute respiratory illnesses (ARIs) including the common cold, bronchiolitis and pneumonia (Turner & Couch, 2007). In addition, recent reports strongly suggest that HRVs may induce exacerbation of wheezing and/or asthma (virus-induced asthma) (Busse *et al.*, 2010; Chung *et al.*, 2007; Khadadah *et al.*, 2010). Thus, these viruses may be associated with ARIs and other severe respiratory illnesses, such as wheezy bronchiolitis and asthma (Busse *et al.*, 2010; Chung *et al.*, 2007; Gern, 2009; Khadadah *et al.*, 2010).

HRVs were previously classified into two species, species A (HRV-A) and B (HRV-B), containing over 100 serotypes (Turner & Couch, 2007). However, a genetically heterogeneous third species, HRV species C (HRV-C), was discovered recently (Lamson *et al.*, 2006; McErlean *et al.*, 2007). Among the species, HRV-A and -C appear to be associated mainly with ARIs and virus-induced asthma, whilst HRV-B has been detected in a relatively small number of patients with ARIs (Linsuwanon *et al.*, 2009; Smuts *et al.*, 2011). Notably, HRV-C species can be detected in most countries and may be associated with various ARIs including upper respiratory infection (URI), bronchiolitis, wheezy bronchiolitis and pneumonia (Smuts *et al.*, 2011; Watanabe *et al.*, 2010), although the epidemiology is not exactly known. However, it is not known whether HRV-A, -B and -C (HRV-ABCs) are associated with severe ARIs such as bronchiolitis, wheezy bronchiolitis and pneumonia. In addition, the epidemiology of HRV-ABCs detected from patients with ARI is unclear in Asian areas, including Japan.

HRV-A and -C may have a wide genetic divergence (Mizuta *et al.*, 2010; Wisdom *et al.*, 2009). Indeed, our previous report indicated that HRV-A strains isolated from Japanese people with various ARIs showed >30 % divergence based on sequences of the *VP4/VP2* coding region and were classified into many clusters by phylogenetic analysis (Mizuta *et al.*, 2010). It has been suggested recently that HRV-ABCs have a unique genetic diversity (McIntyre *et al.*, 2010; Simmonds *et al.*, 2010). In addition, HRV-ABCs may be type assigned using pairwise distances (*p*-distances) (McIntyre *et al.*, 2010; Simmonds *et al.*, 2010). Thus, we applied this method to provisionally type assign the detected strains of HRV-ABCs in the present study. On this basis, we performed a molecular epidemiological study of HRV-ABCs detected in Japanese patients with various ARIs including URI, bronchiolitis, wheezy bronchiolitis, croup and pneumonia.

## METHODS

**Study samples.** A total of 501 nasopharyngeal swab (NPS) samples were collected from patients with ARI. Culture methods and RT-PCR were applied to all samples to detect the various respiratory viruses such as influenza viruses (subtypes A, B and C), parainfluenza viruses (types 1–4), adenoviruses, respiratory syncytial virus, human metapneumovirus and enteroviruses, as well as respiratory bacteria (Echevarría *et al.*, 1998; Miura-Ochiai *et al.*, 2007; Nakauchi *et al.*, 2011; Parveen *et al.*, 2006; Takao *et al.*, 2004). HRV-A, -B or -C alone was detected in 92 of the NPS samples (18.4 %) and genetic analysis of the strains was performed.

**Subjects.** The 92 Japanese patients in whom HRV-A, -B or -C was detected were enrolled in the present study. The samples were obtained by the local health authorities of Tochigi and Yamaguchi prefectures for the surveillance of viral diseases in Japan between January and December 2010. Informed consent was obtained from the subjects, or from the parents of underage subjects, for the donations of samples used in this study. Patients were diagnosed with URI and severe ARIs such as bronchiolitis, wheezy bronchiolitis, croup or pneumonia (Table 1). The diagnosis of ARIs was conducted as described previously (Cherry, 2003; Robert, 2003). All patients were aged 0–36 years (mean ± SD 3.5 ± 5.0 years).

**RNA extraction, RT-PCR and sequencing.** All procedures were performed as described previously (Mizuta *et al.*, 2010). Briefly, for the extraction of viral RNA, RT-PCR and sequence analysis, NPS samples 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 (Mizuta *et al.*, 2010). Viral nucleic acid was extracted from the samples using a QIAamp Viral RNA Mini kit (Qiagen). The reverse transcription reaction mixture was incubated with random hexamers at 42 °C for 90 min, followed by incubation at 99 °C for 5 min and amplification by thermal cycling. Using this cDNA, part of the *VP4/VP2* coding region was amplified by PCR with cycling parameters of 3 min at 94 °C for denaturation, followed by 40 cycles of 94 °C for 30 s, 60 °C for 1 min and 68 °C for 2 min, with a final elongation for 7 min at 68 °C. Purification of DNA fragments and nucleotide sequence determination were performed as described previously (Mizuta *et al.*, 2010). We analysed the nucleotide sequences (nt 623–1012; 390 bp) and the deduced amino acid sequences (130 aa) of the *VP4/VP2* coding region. We took general precautions to prevent any carry-over contamination of PCR, as described previously (Lam *et al.*, 2007).

**Phylogenetic analysis and calculation of *p*-distances.** Phylogenetic analysis of the nucleotide sequences of the partial *VP4/VP2* coding region of HRVs (nt 623–1012) was conducted using the CLUSTAL W program available on the DNA Data Bank of Japan (http://clustalw.ddbj.nig.ac.jp/top-j.html) and Tree Explorer (http://www.megasoftware.net/). Evolutionary distances were estimated according to Kimura's two-parameter method and a phylogenetic tree was reconstructed using the neighbour-joining method (Kimura 1980; Saitou & Nei, 1987). The reliability of the tree was estimated with 1000 bootstrap replications. In addition, to assess interspecies frequency distributions of HRVs, we calculated *p*-distances for all of the strains, including the present strains and reference strains, as described previously (Mizuta *et al.*, 2010).

**Provisional type assignment of HRV-ABCs in the present strains.** It has been suggested that HRV-C strains can be type assigned (Simmonds *et al.*, 2010). In addition, previous reports suggest that, if the correct species *p*-distance values are used, this method may be applicable to the type assignment of HRV-A and -B, as well as HRV-C, on the phylogenetic trees (McIntyre *et al.*, 2010; Simmonds *et al.*, 2010). Thus, we thought that it would be possible to conduct type assignment of the present strains (HRV-ABCs). In this study, *VP4/VP2* sequences that were available and which showed >10 % divergence from other species in the sequences in this region were provisionally type assigned.

**Table 1.** Subject data in this study

| Species | No. strains | Sex (male/female) | Age (year)* | Clinical symptoms | No. patients |
|---|---|---|---|---|---|
| HRV-A | 47 | 27/20 | 3.0±5.3 | URI | 20 |
| | | | | Bronchiolitis | 19 |
| | | | | Wheezy bronchiolitis | 5 |
| | | | | Croup | 1 |
| | | | | Pneumonia | 2 |
| HRV-B | 2 | 1/1 | 13.5±13.4 | URI | 1 |
| | | | | Bronchiolitis | 1 |
| HRV-C | 43 | 25/18 | 3.6±3.0 | URI | 9 |
| | | | | Bronchiolitis | 6 |
| | | | | Wheezy bronchiolitis | 25 |
| | | | | Pneumonia | 3 |
| Total | 92 | 53/39 | 3.5±5.0 | URI | 30 |
| | | | | Bronchiolitis | 26 |
| | | | | Wheezy bronchiolitis | 30 |
| | | | | Croup | 1 |
| | | | | Pneumonia | 5 |

*Data are expressed as mean±SD.

## RESULTS

### Seasonal variation of HRV-ABCs

HRV-ABCs were detected in 92 of 501 NPS samples from patients with ARIs (18.4%). HRV-A and -C were detected throughout the investigation period (Fig. 1). However, the monthly distribution differed among the species: HRV-A and -C were predominantly detected in April–June and September–December, respectively, whilst HRV-B was detected sporadically. HRV-A strains were the most prevalent (47/92; 51.1%), followed by HRV-C (43/92; 46.7%) and HRV-B (2/92; 2.2%).

### Phylogenetic analysis, provisional type assignment and homology analysis

First, we reconstructed phylogenetic trees based on the nucleotide (390 nt) and deduced amino acid (130 aa) sequences of HRV-A and -C with regard to the reference strains (Fig. 2a–d). Using these reference strains and the present strains, we then reconstructed phylogenetic trees based on the nucleotide sequences and deduced amino acid sequences of the *VP4/VP2* coding region (Fig. 2e, f). Based on the nucleotide sequences, HRV-A, -B and -C were provisionally assigned to 14, 2 and 12 types, respectively, in the phylogenetic trees. The number of HRV-A strains in each type in the phylogenetic tree based on the nucleotide sequences was as follows: HRV45, three strains; HRV7, five strains; HRV46, one strain; HRV53, one strain; HRV20, four strains; HRV12, seven strains; HRV23, two strains; HRV40, six strains; HRV59, three strains; HRV76, two strains; HRV11, two strains; HRV19, one strain; HRV10, three strains and HRV16, seven strains. Two HRV-B strains were provisionally assigned to HRV3 and HRV17 based on the nucleotide sequences. The number of HRV-C strains in each type, based on nucleotide sequences, was as follows: g2-11 (HRV-C39), five strains; SA365412 (pat14), six strains; tu304 (HRV-C38), one strain; IN-36 (HRV-C49), one strain; g2-25 (HRV-C40), two strains; RV546 (pat18), two strains; Cd08-1009 U (pat28), one strain and g2-23 (HRV-C41), eight strains. In addition, four possibly unique types of HRV-C strains were found. The number of HRV-C strains assigned to each of these types was as follows: not typed (A) (HRV/Yamaguchi/2010/YU64 type), five strains; not typed (B) (HRV/Yamaguchi/2010/YU42 type), one strain; not typed (C) (HRV/Tochigi/2010/353), one strain; and not typed (D) (HRV/Yamaguchi/2010/YU74 type), 10 strains. The HRV-A and -C strains detected in most types were associated with bronchiolitis and/or wheezy bronchiolitis.

At the nucleotide level, the identity among the detected strains was 63.3–100% (HRV-A), 66.2% (HRV-B) and 63.4–100% (HRV-C), whilst at the deduced amino acid level the identity was 85.4–100% (HRV-A), 97.1% (HRV-B) and 89.5–100% (HRV-C). These results suggest that the present HRV-ABCs had >30% nucleotide divergence [63.3–100% (HRV-A), 66.2% (HRV-B) and 63.4–100% (HRV-C) identity].

### Interspecies *p*-distances of HRVs

We calculated the interspecies distances of HRVs by the distribution of *p*-distances (Figs 3 and 4). Among the present and reference strains, the interspecies distances based on the nucleotide sequences were 0.230±0.063 (mean±SD, HRV-A), 0.218±0.048 (HRV-B) and 0.281±0.105 (HRV-C), and 0.075±0.036 (HRV-A), 0.049±0.022 (HRV-B) and 0.141±0.064 (HRV-C) at the deduced amino acid level. HRV-C strains had the longest interspecies *p*-distances based on the nucleotide and amino acid sequences.

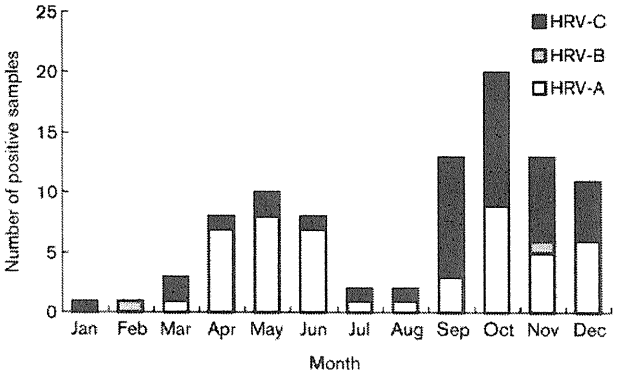



**Fig. 1.** Seasonal variations of HRV-ABCs.

## DISCUSSION

In this study, we detected HRV-ABCs from 92 Japanese patients with various ARIs such as URI, bronchiolitis, wheezy bronchiolitis, croup and pneumonia between January and December 2010. Based on the nucleotide sequences of the *VP4/VP2* coding region, HRV-A and -C strains showed a wide genetic diversity (>30 % divergence) and were classified into many types in the phylogenetic tree (Fig. 2). However, we detected HRV-B from only two cases of ARI. These results suggested that there are various types of HRV-A and -C strains, which may be associated with various ARIs in Japan.

Wisdom *et al.* (2009) suggested that HRV-A and -C are the predominant strains detected in patients with ARIs in the UK. Moreover, these strains showed a wide genetic diversity and were classified into many types in the phylogenetic tree (Wisdom *et al.*, 2009). Smuts *et al.* (2011) demonstrated that HRV-ABCs were the predominant strains detected in African children with acute wheezing. In addition, our previous report suggested that HRV-A isolates from children with ARIs (in Yamagata prefecture, Japan) were classified into 11 clusters in the phylogenetic tree with >30 % nucleotide divergence of the *VP4/VP2* coding region (Mizuta *et al.*, 2010). These results suggest that, in some countries, HRV-A and -C detected in ARI cases are the predominant strains and have varied genetic properties (Mizuta *et al.*, 2010; Wisdom *et al.*, 2009). We then calculated the interspecies distances and divergence of the strains and compared the results with previous reports. HRV-A had similar interspecies distances and divergence (Mizuta *et al.*, 2010). In addition, the *p*-distances between HRV-C strains ($0.281 \pm 0.105$) were greater than those between HRV-A strains ($0.230 \pm 0.063$). Our present results thus appeared to be compatible with these earlier reports (McIntyre *et al.*, 2010; Mizuta *et al.*, 2010; Simmonds *et al.*, 2010).

It has been suggested recently that the correct type assignment of HRV-C strains is possible if based on the *p*-distances within HRV-C (McIntyre *et al.*, 2010; Simmonds *et al.*, 2010). In these reports, *VP4/VP2* coding regions that are available and that show <10 % divergence from other HRV-C species sequences in the region may be provisionally type assigned (Simmonds *et al.*, 2010). Another report suggested that divergence of the *VP4/VP2* coding regions may not differ significantly among HRV-ABCs, although the divergence of HRV-C may be greater than that of HRV-A and -B (Simmonds *et al.*, 2010). To the best of our knowledge, it is not known whether this method is applicable to the type assignment of other HRV species, such as HRV-A and -B. However, previous reports suggest that, if the correct species *p*-distance values are used, this method may also be applicable to the type assignment of HRV-A and -B in the phylogenetic trees (McIntyre *et al.*, 2010; Simmonds *et al.*, 2010). From these findings and our data, we carefully selected suitable reference strains for the type assignment of HRV-ABCs. Finally, through these processes, the present strains were provisionally assigned to various types in the phylogenetic trees. As a result, HRV-A, -B and -C in the phylogenetic trees in this study were classified into 14, 2 and 12 types, respectively. Notably, four unique types of HRV-C strain were found. Further studies may be needed to assign HRV-A and -B strains with certainty using more detailed genetic data.

HRVs were previously thought to be associated mainly with the common cold, producing mild respiratory symptoms (Gern, 2010; Turner & Couch, 2007). For this reason, the optimum propagation temperature of HRVs may be 32–35 °C *in vitro* (Papadopoulos *et al.*, 1999; Schroth *et al.*, 1999). However, a recent study suggested that HRVs can propagate in lower airway tissues and this may be an important factor in the development of airway obstruction, coughing and wheezing that can lead to bronchiolitis and pneumonia (Mosser *et al.*, 2005). HRVs are being re-evaluated as an important agent of ARIs in humans (Imakita *et al.*, 2000; Papadopoulos *et al.*, 2002; Wos *et al.*, 2008). A very recent study suggested that HRVs are a major agent in the induction of wheezing and exacerbation of asthma (Khadadah *et al.*, 2010). In the present cases, HRV-A and -C were detected from patients with severe ARIs such as bronchiolitis, wheezy bronchiolitis, croup and pneumonia. In addition, another recent study has suggested a novel organ culture method to enable the propagation of HRV-C (Bochkov *et al.*, 2011), although this species could not be isolated by previous cell-culture methods. Thus, details of the pathogenesis and epidemiology of HRV-ABCs may be elucidated in the future.

The viruses detected during the investigation period, as illustrated in Fig. 1, showed the seasonal variations of HRVs in Japan. HRV-B strains were detected twice, in February and November, whilst HRV-A and -C were the predominant species and were detected throughout the period. A recent study suggested that HRV-C has a stronger link to severe respiratory illness, such as virus-induced asthma, than HRV-A and -B strains (Miller *et al.*, 2009). Indeed, our results revealed a similar significance ($P<0.05$). Thus, HRV-ABCs might be associated with

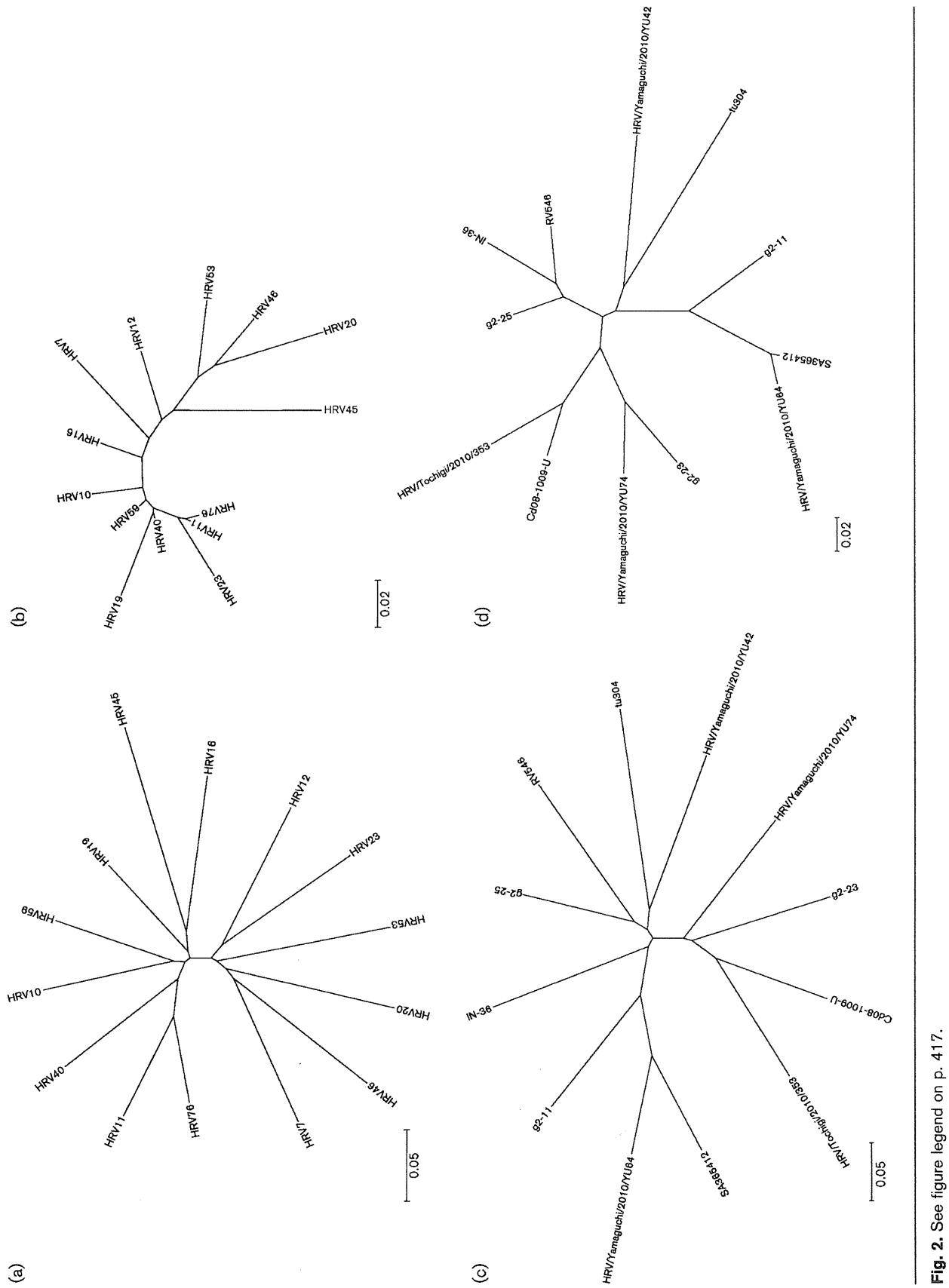
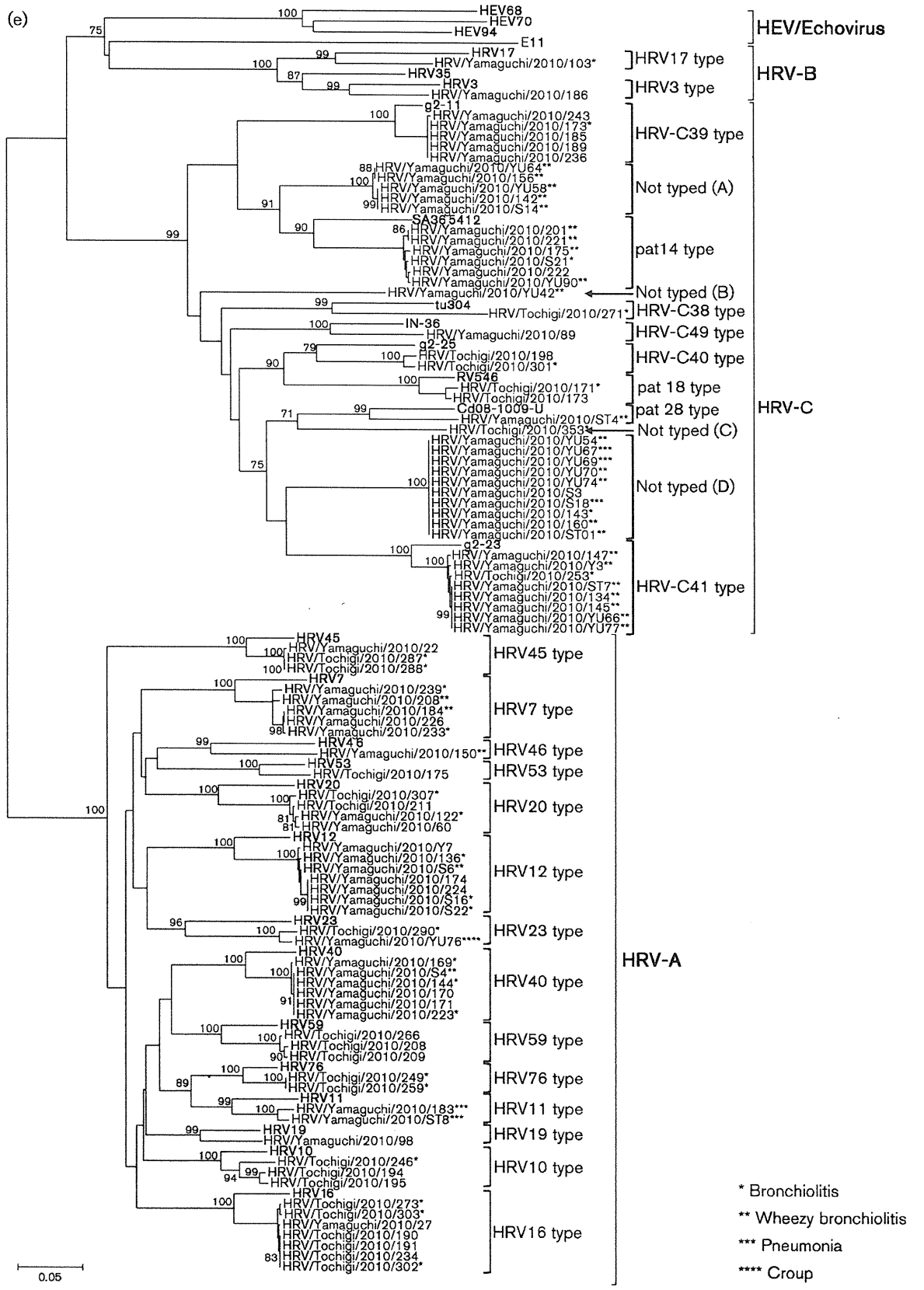



**Fig. 2.** See figure legend on p. 417.


(e)
HEV68
HEV70
HEV94
E11
HRV17
HRV/Yamaguchi/2010/103*
HRV35
HRV3
HRV/Yamaguchi/2010/186
g2-11
HRV/Yamaguchi/2010/243
HRV/Yamaguchi/2010/173*
HRV/Yamaguchi/2010/185
HRV/Yamaguchi/2010/189
HRV/Yamaguchi/2010/236
HRV/Yamaguchi/2010/YU64**
HRV/Yamaguchi/2010/156**
HRV/Yamaguchi/2010/YU58**
HRV/Yamaguchi/2010/142**
HRV/Yamaguchi/2010/S14**
SA36-5412
HRV/Yamaguchi/2010/201**
HRV/Yamaguchi/2010/221**
HRV/Yamaguchi/2010/175**
HRV/Yamaguchi/2010/S21*
HRV/Yamaguchi/2010/222
HRV/Yamaguchi/2010/YU90**
HRV/Yamaguchi/2010/YU42**
tu304
HRV/Tochigi/2010/271*
IN-36
HRV/Yamaguchi/2010/89
g2-25
HRV/Tochigi/2010/198
HRV/Tochigi/2010/301*
RV546
HRV/Tochigi/2010/171*
HRV/Tochigi/2010/173
Cd08-1009-U
HRV/Yamaguchi/2010/ST4**
HRV/Tochigi/2010/353*
HRV/Yamaguchi/2010/YU54**
HRV/Yamaguchi/2010/YU67***
HRV/Yamaguchi/2010/YU69***
HRV/Yamaguchi/2010/YU70**
HRV/Yamaguchi/2010/YU74**
HRV/Yamaguchi/2010/S3
HRV/Yamaguchi/2010/S18***
HRV/Yamaguchi/2010/143*
HRV/Yamaguchi/2010/160**
HRV/Yamaguchi/2010/ST01**
g2-23
HRV/Yamaguchi/2010/147**
HRV/Yamaguchi/2010/Y3**
HRV/Tochigi/2010/253*
HRV/Yamaguchi/2010/ST7**
HRV/Yamaguchi/2010/134**
HRV/Yamaguchi/2010/145**
HRV/Yamaguchi/2010/YU66**
HRV/Yamaguchi/2010/YU77**
HRV45
HRV/Yamaguchi/2010/22
HRV/Tochigi/2010/287*
HRV/Tochigi/2010/288*
HRV7
HRV/Yamaguchi/2010/239*
HRV/Yamaguchi/2010/208**
HRV/Yamaguchi/2010/184**
HRV/Yamaguchi/2010/226
HRV/Yamaguchi/2010/233*
HRV46
HRV/Yamaguchi/2010/150**
HRV53
HRV/Tochigi/2010/175
HRV20
HRV/Tochigi/2010/307*
HRV/Tochigi/2010/211
HRV/Yamaguchi/2010/122*
HRV/Yamaguchi/2010/60
HRV12
HRV/Yamaguchi/2010/Y7
HRV/Yamaguchi/2010/136*
HRV/Yamaguchi/2010/S6**
HRV/Yamaguchi/2010/174
HRV/Yamaguchi/2010/224
HRV/Yamaguchi/2010/S16*
HRV/Yamaguchi/2010/S22*
HRV23
HRV/Tochigi/2010/290*
HRV/Yamaguchi/2010/YU76****
HRV40
HRV/Yamaguchi/2010/169*
HRV/Yamaguchi/2010/S4**
HRV/Yamaguchi/2010/144*
HRV/Yamaguchi/2010/170
HRV/Yamaguchi/2010/171
HRV/Yamaguchi/2010/223*
HRV59
HRV/Tochigi/2010/266
HRV/Tochigi/2010/208
HRV/Tochigi/2010/209
HRV76
HRV/Tochigi/2010/249*
HRV/Tochigi/2010/259*
HRV11
HRV/Yamaguchi/2010/183***
HRV/Yamaguchi/2010/ST8***
HRV19
HRV/Yamaguchi/2010/98
HRV10
HRV/Tochigi/2010/246*
HRV/Tochigi/2010/194
HRV/Tochigi/2010/195
HRV16
HRV/Tochigi/2010/273*
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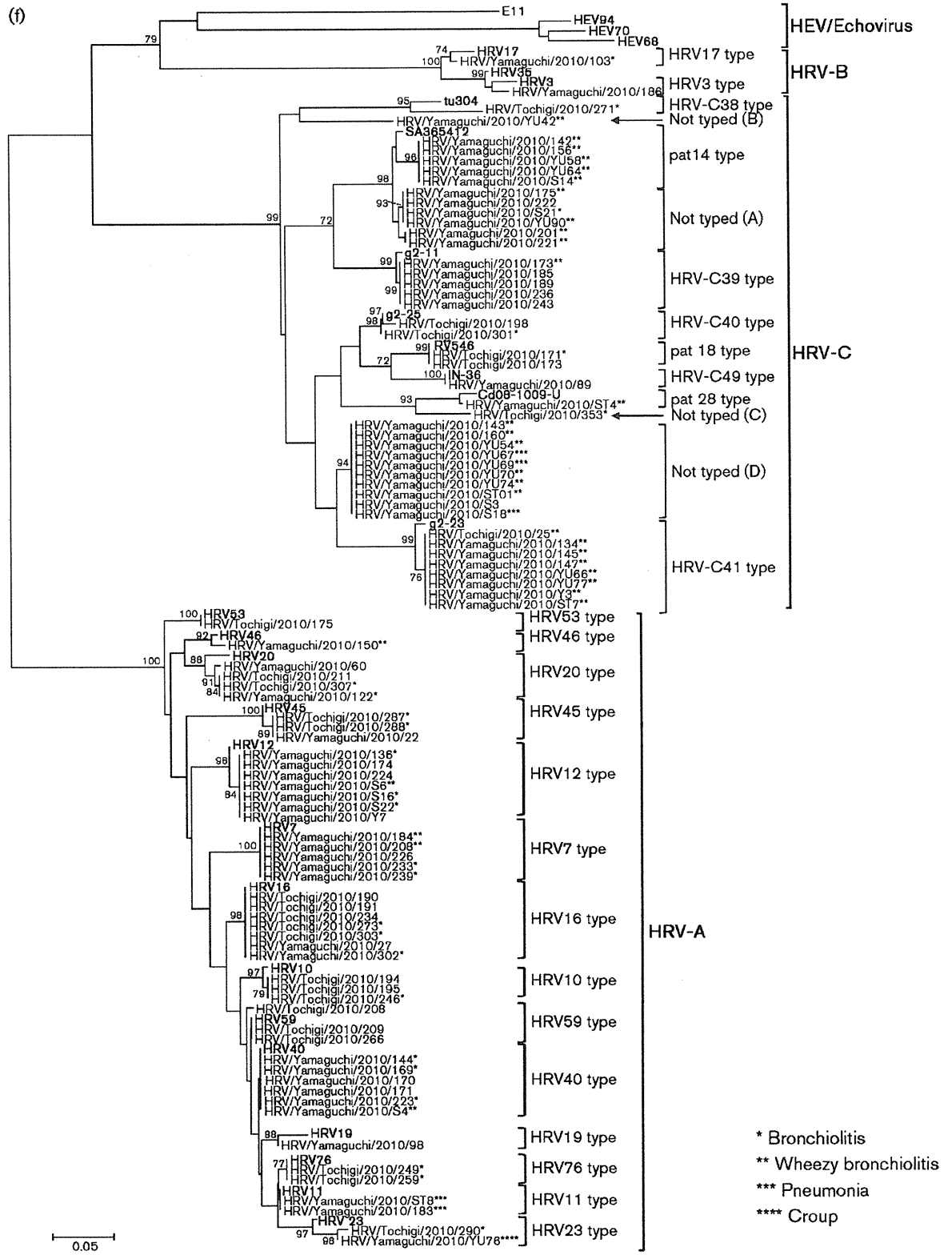
HRV/Tochigi/2010/302*
0.05
HEV/Echovirus
HRV-B
HRV17 type
HRV3 type
HRV-C
HRV-C39 type
Not typed (A)
pat14 type
Not typed (B)
HRV-C38 type
HRV-C49 type
HRV-C40 type
pat 18 type
pat 28 type
Not typed (C)
Not typed (D)
HRV-C41 type
HRV-A
HRV45 type
HRV7 type
HRV46 type
HRV53 type
HRV20 type
HRV12 type
HRV23 type
HRV40 type
HRV59 type
HRV76 type
HRV11 type
HRV19 type
HRV10 type
HRV16 type
* Bronchiolitis
** Wheezy bronchiolitis
*** Pneumonia
**** Croup


(f)
E11
HEV94
HEV70
HEV68
HEV/Echovirus
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HRV/Yamaguchi/2010/186
HRV3 type
HRV-B
tu304
HRV/Tochigi/2010/271*
HRV-C38 type
HRV/Yamaguchi/2010/YU42**
Not typed (B)
SA365412
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HRV/Yamaguchi/2010/YU64**
HRV/Yamaguchi/2010/S14**
pat14 type
HRV/Yamaguchi/2010/175**
HRV/Yamaguchi/2010/222
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HRV/Yamaguchi/2010/YU90**
HRV/Yamaguchi/2010/201**
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92-11
HRV/Yamaguchi/2010/173**
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HRV/Yamaguchi/2010/236
HRV/Yamaguchi/2010/243
HRV-C39 type
92-25
HRV/Tochigi/2010/198
HRV/Tochigi/2010/301*
HRV-C40 type
RV546
HRV/Tochigi/2010/171*
HRV/Tochigi/2010/173
pat 18 type
HRV-C
IN-36
HRV/Yamaguchi/2010/89
HRV-C49 type
Cd08-1009-U
HRV/Yamaguchi/2010/ST4**
pat 28 type
HRV/Tochigi/2010/353*
Not typed (C)
HRV/Yamaguchi/2010/143**
HRV/Yamaguchi/2010/160**
HRV/Yamaguchi/2010/YU54**
HRV/Yamaguchi/2010/YU67***
HRV/Yamaguchi/2010/YU69***
HRV/Yamaguchi/2010/YU70**
HRV/Yamaguchi/2010/YU74**
HRV/Yamaguchi/2010/ST01**
HRV/Yamaguchi/2010/S3
HRV/Yamaguchi/2010/S18***
Not typed (D)
92-23
HRV/Tochigi/2010/25**
HRV/Yamaguchi/2010/134**
HRV/Yamaguchi/2010/145**
HRV/Yamaguchi/2010/147**
HRV/Yamaguchi/2010/YU66**
HRV/Yamaguchi/2010/YU77**
HRV/Yamaguchi/2010/Y3**
HRV/Yamaguchi/2010/ST7**
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HRV53 type
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HRV/Yamaguchi/2010/150**
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HRV/Yamaguchi/2010/136*
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HRV/Yamaguchi/2010/224
HRV/Yamaguchi/2010/S6**
HRV/Yamaguchi/2010/S16*
HRV/Yamaguchi/2010/S22*
HRV/Yamaguchi/2010/Y7
HRV12 type
HRV7
HRV/Yamaguchi/2010/184**
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HRV/Yamaguchi/2010/226
HRV/Yamaguchi/2010/233*
HRV/Yamaguchi/2010/239*
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HRV/Tochigi/2010/303*
HRV/Yamaguchi/2010/27
HRV/Yamaguchi/2010/302*
HRV16 type
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HRV10
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HRV/Tochigi/2010/195
HRV/Tochigi/2010/246*
HRV10 type
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HRV59
HRV/Tochigi/2010/209
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HRV59 type
HRV40
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HRV/Yamaguchi/2010/169*
HRV/Yamaguchi/2010/170
HRV/Yamaguchi/2010/171
HRV/Yamaguchi/2010/223*
HRV/Yamaguchi/2010/S4**
HRV40 type
HRV19
HRV/Yamaguchi/2010/98
HRV19 type
HRV76
HRV/Tochigi/2010/249*
HRV/Tochigi/2010/259*
HRV76 type
HRV11
HRV/Yamaguchi/2010/ST8***
HRV/Yamaguchi/2010/183***
HRV11 type
HRV 23
HRV/Tochigi/2010/290*
HRV/Yamaguchi/2010/YU76****
HRV23 type
0.05
* Bronchiolitis
** Wheezy bronchiolitis
*** Pneumonia
**** Croup

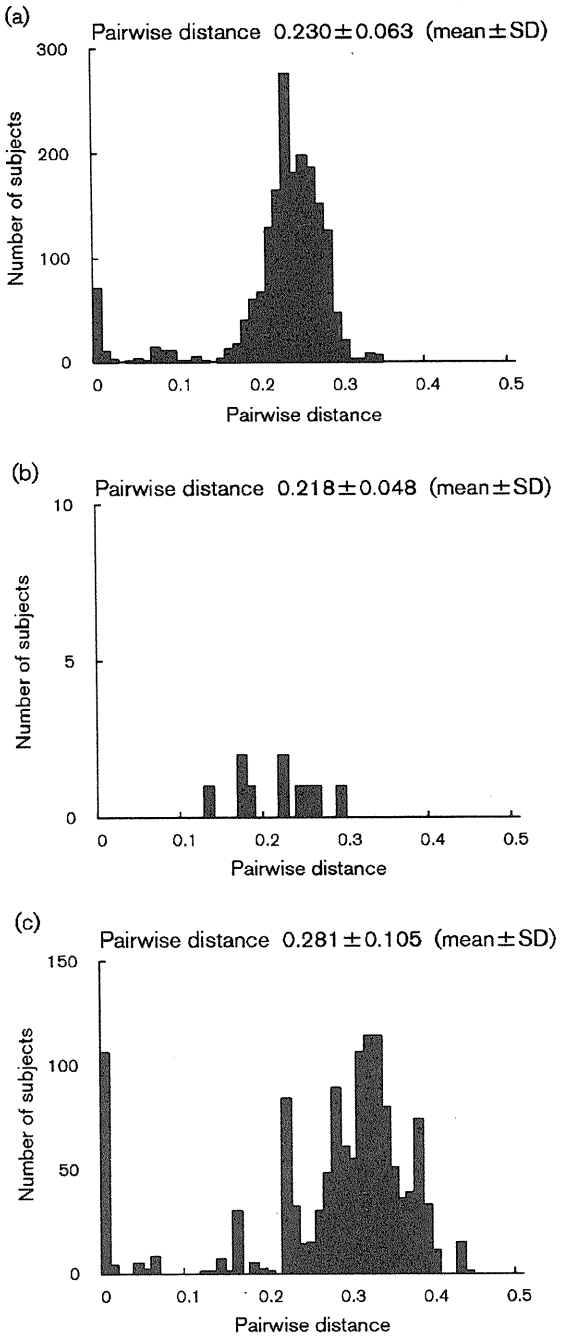


**Fig. 3.** Distributions of pairwise interspecies distances for HRV-A (a), HRV-B (b) and HRV-C (c) based on the nucleotide sequences of the *VP4/VP2* coding region.

**Fig. 2.** Phylogenetic trees based on the *VP4/VP2* coding region (390 bp) of HRV-ABCs. (a–e) Phylogenetic trees based on the nucleotide sequences (a) and deduced amino acid sequences (b) of HRV-A reference strains, the nucleotide sequences (c) and deduced amino acid sequences (d) of HRV-C reference strains, and the nucleotide sequences of the *VP4/VP2* coding region of HRV-ABCs and human enterovirus species D (HEV-D) (e). The reference strains used for type assignment were obtained from Picornaviridae.com (http://www.picornaviridae.com/). The following reference strains were used: HRV3, HRV7, HRV10, HRV11, HRV12, HRV16, HRV17, HRV19, HRV20, HRV23, HRV35, HRV40, HRV45, HRV46, HRV53, HRV59, HRV76, g2-11 (HRV-C39), tu304 (HRV-C38), IN-36 (HRV-C49), g2-25 (HRV-C40), g2-23 (HRV-C41), SA365412 (pat14), RV546 (pat18) and Cd08-1009 U (pat28), Echovirus 11 (E11), which belongs to the human enterovirus species B, was used as an outgroup. Bars, substitutions per nucleotide position. (f) Phylogenetic tree of the deduced amino acid sequences of the *VP4/VP2* coding region (130 aa), including the present strains and representative reference strains. The tree was reconstructed by neighbour-joining analysis with labelling of the branches showing ⩾ 70 % bootstrap support. Bar, substitutions per amino acid position.

severe ARIs such as bronchiolitis, wheezy bronchiolitis, croup and pneumonia throughout the seasons in Japan.

This study used samples obtained from ARI patients who tested negative for other respiratory viruses, but they were not tested for any additional viruses and therefore dual

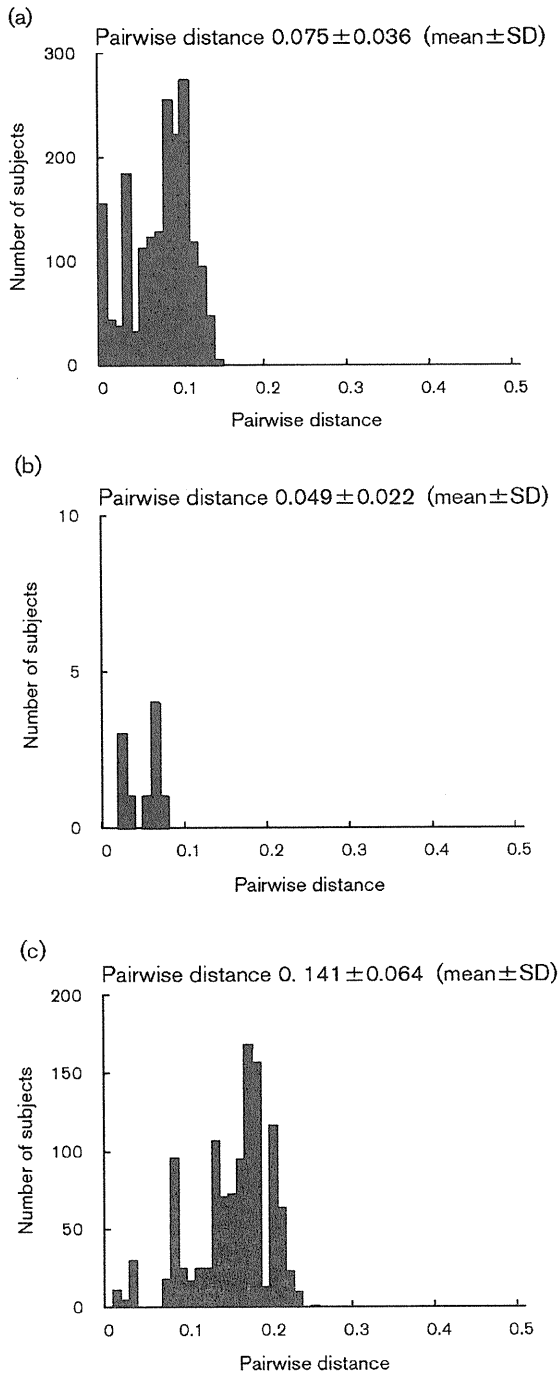


**Fig. 4.** Distributions of pairwise interspecies distances for HRV-A (a), HRV-B (b) and HRV-C (c) based on the deduced amino acid sequences of the *VP4/VP2* coding region.

infection cannot be excluded. In addition, we cannot exclude the possibility that HRVs are present asymptomatically in humans, because samples from healthy persons were not tested.

In conclusion, various genetic types of HRV-ABCs appear to be associated with ARIs, including URI, wheezy bronchiolitis, croup and pneumonia, in the Japanese population. Further and larger studies are warranted to address the detailed genetic properties of HRVs.

## ACKNOWLEDGEMENTS

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## REFERENCES

**Bochkov, Y. A., Palmenberg, A. C., Lee, W.-M., Rathe, J. A., Amineva, S. P., Sun, X., Pasic, T. R., Jarjour, N. N., Liggett, S. B. & Gern, J. E. (2011).** Molecular modeling, organ culture and reverse genetics for a newly identified human rhinovirus C. *Nat Med* **17**, 627–632.

**Busse, W. W., Lemanske, R. F., Jr & Gern, J. E. (2010).** Role of viral respiratory infections in asthma and asthma exacerbations. *Lancet* **376**, 826–834.

**Cherry, J. D. (2003).** The Common Cold. In *Textbook of Pediatric Infectious Diseases*, 5th edn, pp. 140–146. Edited by R. D. Feigin, J. D. Cherry, G. J. Demmler & S. L. Kaplan. Philadelphia: Saunders.

**Chung, J.-Y., Han, T. H., Kim, S. W., Kim, C. K. & Hwang, E.-S. (2007).** Detection of viruses identified recently in children with acute wheezing. *J Med Virol* **79**, 1238–1243.

**Echevarría, J. E., Erdman, D. D., Swierkosz, E. M., Holloway, B. P. & Anderson, L. J. (1998).** Simultaneous detection and identification of human parainfluenza viruses 1, 2, and 3 from clinical samples by multiplex PCR. *J Clin Microbiol* **36**, 1388–1391.

**Gern, J. E. (2009).** Rhinovirus and the initiation of asthma. *Curr Opin Allergy Clin Immunol* **9**, 73–78.

**Gern, J. E. (2010).** The ABCs of rhinoviruses, wheezing, and asthma. *J Virol* **84**, 7418–7426.

**Imakita, M., Shiraki, K., Yutani, C. & Ishibashi-Ueda, H. (2000).** Pneumonia caused by rhinovirus. *Clin Infect Dis* **30**, 611–612.

**Khadadah, M., Essa, S., Higazi, Z., Behbehani, N. & Al-Nakib, W. (2010).** Respiratory syncytial virus and human rhinoviruses are the major causes of severe lower respiratory tract infections in Kuwait. *J Med Virol* **82**, 1462–1467.

**Kimura, M. (1980).** A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.

**Lam, W. Y., Yeung, A. C., Tang, J. W., Ip, M., Chan, E. W., Hui, M. & Chan, P. K. (2007).** Rapid multiplex nested PCR for detection of respiratory viruses. *J Clin Microbiol* **45**, 3631–3640.

**Lamson, D., Renwick, N., Kapoor, V., Liu, Z., Palacios, G., Ju, J., Dean, A., St George, K., Briese, T. & Lipkin, W. I. (2006).** MassTag polymerase-chain-reaction detection of respiratory pathogens, including a new rhinovirus genotype, that caused influenza-like illness in New York State during 2004–2005. *J Infect Dis* **194**, 1398–1402.

**Linsuwanon, P., Payungporn, S., Samransamruajkit, R., Posuwan, N., Makkoch, J., Theanboonlers, A. & Poovorawan, Y. (2009).** High prevalence of human rhinovirus C infection in Thai children with acute lower respiratory tract disease. *J Infect* **59**, 115–121.

**McErlean, P., Shackelton, L. A., Lambert, S. B., Nissen, M. D., Sloots, T. P. & Mackay, I. M. (2007).** Characterisation of a newly identified

human rhinovirus, HRV-QPM, discovered in infants with bronchiolitis. *J Clin Virol* **39**, 67–75.

**McIntyre, C. L., McWilliam Leitch, E. C., Savolainen-Kopra, C., Hovi, T. & Simmonds, P. (2010).** Analysis of genetic diversity and sites of recombination in human rhinovirus species C. *J Virol* **84**, 10297–10310.

**Miller, E. K., Edwards, K. M., Weinberg, G. A., Iwane, M. K., Griffin, M. R., Hall, C. B., Zhu, Y., Szilagyi, P. G., Morin, L. L. & other authors (2009).** A novel group of rhinoviruses is associated with asthma hospitalizations. *J Allergy Clin Immunol* **123**, 98–104.e1.

**Miura-Ochiai, R., Shimada, Y., Konno, T., Yamazaki, S., Aoki, K., Ohno, S., Suzuki, E. & Ishiko, H. (2007).** Quantitative detection and rapid identification of human adenoviruses. *J Clin Microbiol* **45**, 958–967.

**Mizuta, K., Hirata, A., Suto, A., Aoki, Y., Ahiko, T., Itagaki, T., Tsukagoshi, H., Morita, Y., Obuchi, M. & other authors (2010).** Phylogenetic and cluster analysis of human rhinovirus species A (HRV-A) isolated from children with acute respiratory infections in Yamagata, Japan. *Virus Res* **147**, 265–274.

**Mosser, A. G., Vrtis, R., Burchell, L., Lee, W.-M., Dick, C. R., Weisshaar, E., Bock, D., Swenson, C. A., Cornwell, R. D. & other authors (2005).** Quantitative and qualitative analysis of rhinovirus infection in bronchial tissues. *Am J Respir Crit Care Med* **171**, 645–651.

**Nakauchi, M., Yasui, Y., Miyoshi, T., Minagawa, H., Tanaka, T., Tashiro, M. & Kageyama, T. (2011).** One-step real-time reverse transcription-PCR assays for detecting and subtyping pandemic influenza A/H1N1 2009, seasonal influenza A/H1N1, and seasonal influenza A/H3N2 viruses. *J Virol Methods* **171**, 156–162.

**Papadopoulos, N. G., Sanderson, G., Hunter, J. & Johnston, S. L. (1999).** Rhinoviruses replicate effectively at lower airway temperatures. *J Med Virol* **58**, 100–104.

**Papadopoulos, N. G., Moustaki, M., Tsolia, M., Bossios, A., Astra, E., Prezerakou, A., Gourgiotis, D. & Kafetzis, D. (2002).** Association of rhinovirus infection with increased disease severity in acute bronchiolitis. *Am J Respir Crit Care Med* **165**, 1285–1289.

**Parveen, S., Sullender, W. M., Fowler, K., Lefkowitz, E. J., Kapoor, S. K. & Broor, S. (2006).** Genetic variability in the G protein gene of group A and B respiratory syncytial viruses from India. *J Clin Microbiol* **44**, 3055–3064.

**Robert, C. W. (2003).** Bronchiolitis and infectious asthma. In *Textbook of Pediatric Infectious Diseases*, 5th edn, pp. 273–282. Edited by R. D. Feigin, J. D. Cherry, G. J. Demmler & S. L. Kaplan. Philadelphia: Saunders.

**Saitou, N. & Nei, M. (1987).** The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

**Schroth, M. K., Grimm, E., Frindt, P., Galagan, D. M., Konno, S. I., Love, R. & Gern, J. E. (1999).** Rhinovirus replication causes RANTES production in primary bronchial epithelial cells. *Am J Respir Cell Mol Biol* **20**, 1220–1228.

**Simmonds, P., McIntyre, C., Savolainen-Kopra, C., Tapparel, C., Mackay, I. M. & Hovi, T. (2010).** Proposals for the classification of human rhinovirus species C into genotypically assigned types. *J Gen Virol* **91**, 2409–2419.

**Smuts, H. E., Workman, L. J. & Zar, H. J. (2011).** Human rhinovirus infection in young African children with acute wheezing. *BMC Infect Dis* **11**, 65.

**Takao, S., Shimozono, H., Kashiwa, H., Matsubara, K., Sakano, T., Ikeda, M., Okamoto, N., Yoshida, H., Shimazu, Y. & Fukuda, S. (2004).** [The first report of an epidemic of human metapneumovirus infection in Japan: clinical and epidemiological study]. *Kansenshogaku Zasshi* **78**, 129–137 (in Japanese).

**Turner, R. B. & Couch, R. B. (2007).** Rhinovirus. In *Fields Virology*, 5th edn, pp. 895–909. Edited by D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman & S. E. Straus. Philadelphia: Lippincott Williams & Wilkins.

**Watanabe, A., Carraro, E., Kamikawa, J., Leal, E., Granato, C. & Bellei, N. (2010).** Rhinovirus species and their clinical presentation among different risk groups of non-hospitalized patients. *J Med Virol* **82**, 2110–2115.

**Wisdom, A., Leitch, E. C., Gaunt, E., Harvala, H. & Simmonds, P. (2009).** Screening respiratory samples for detection of human rhinoviruses (HRVs) and enteroviruses: comprehensive VP4–VP2 typing reveals high incidence and genetic diversity of HRV species C. *J Clin Microbiol* **47**, 3958–3967.

**Wos, M., Sanak, M., Soja, J., Olechnowicz, H., Busse, W. W. & Szczeklik, A. (2008).** The presence of rhinovirus in lower airways of patients with bronchial asthma. *Am J Respir Crit Care Med* **177**, 1082–1089.