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Additional files

Additional_file_1 as DOC
Additional file 1. Table S1. Subject data in this study.

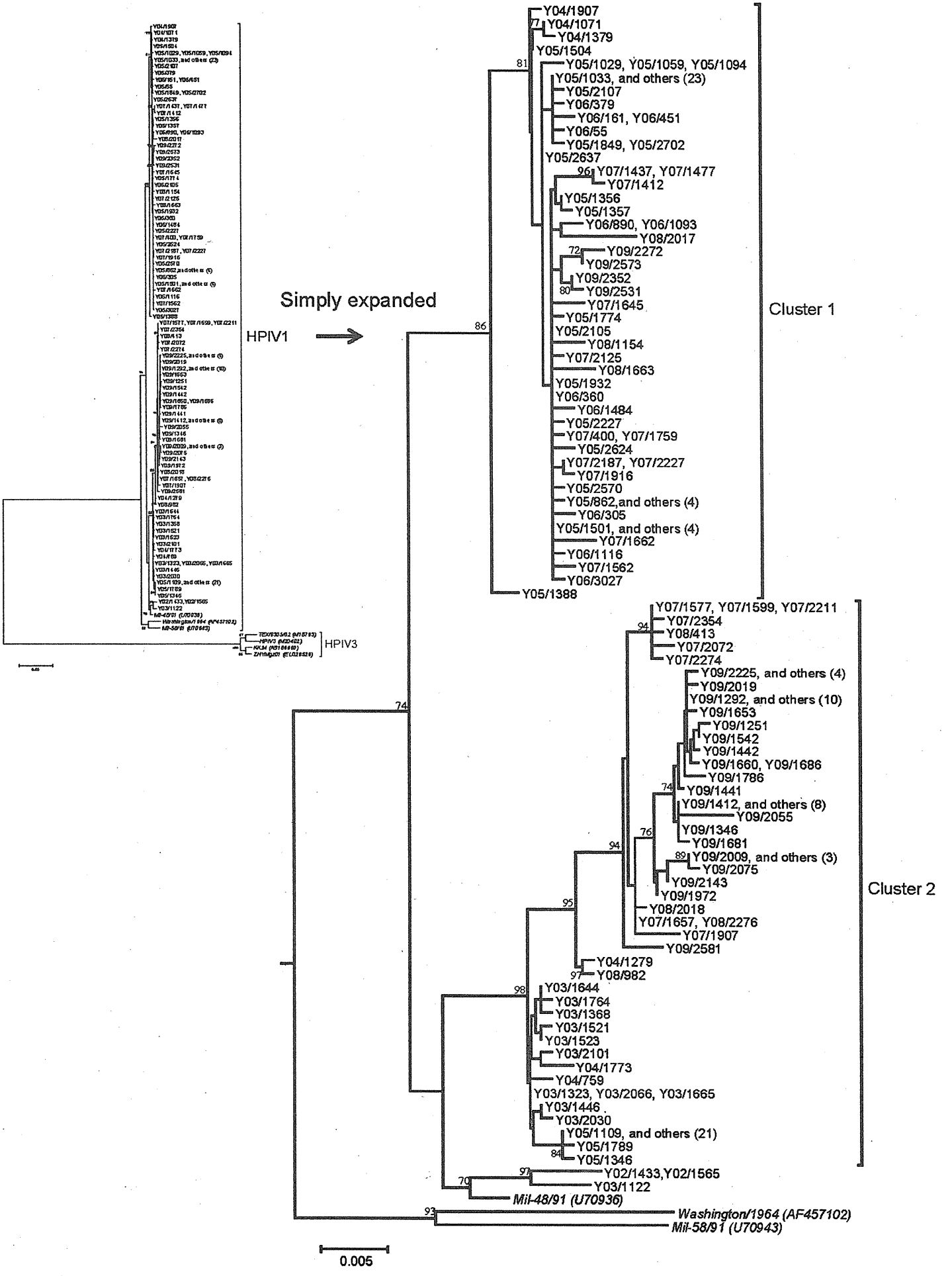
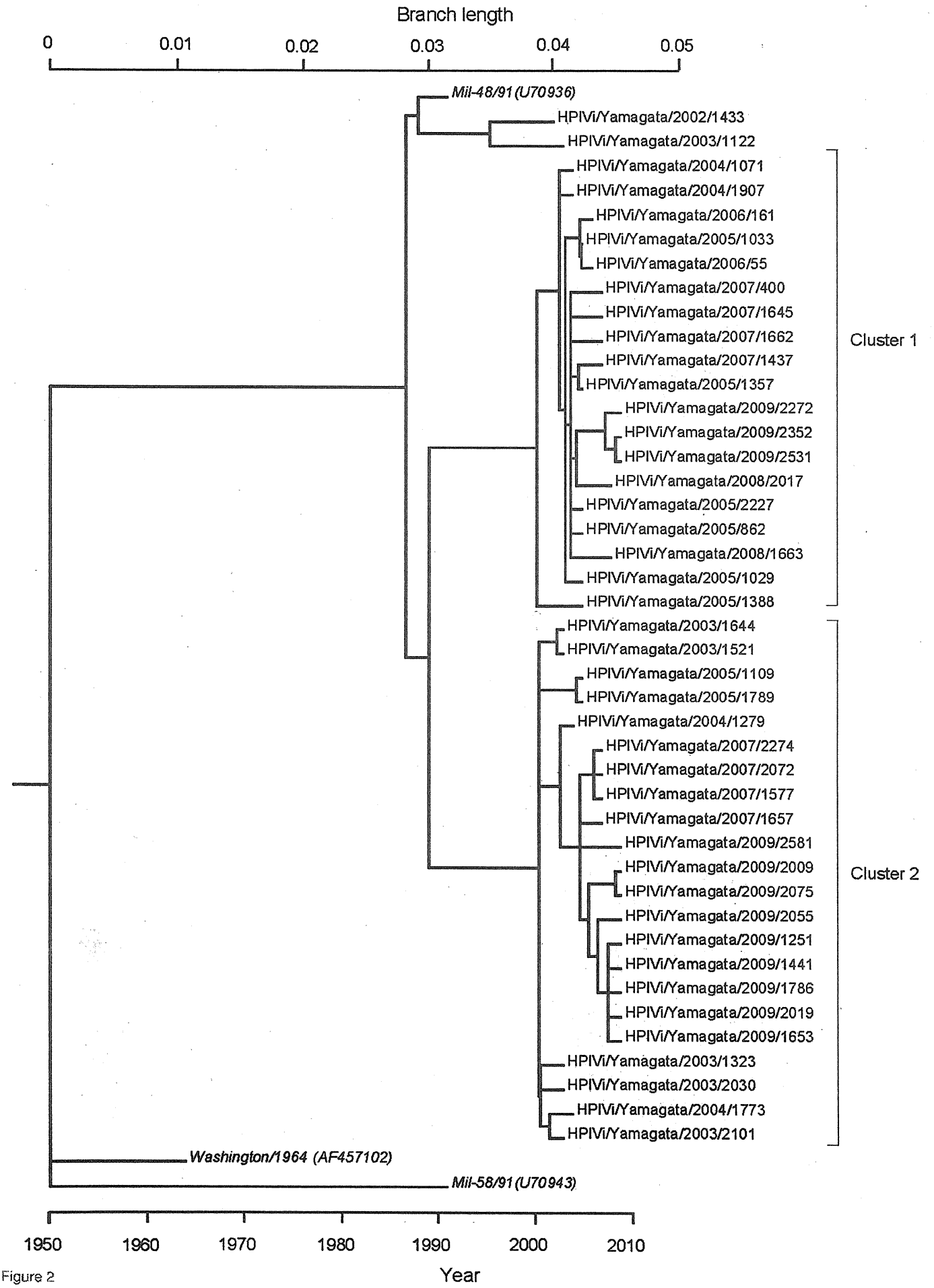

Simply expanded
HPIV1
HPIV3
Cluster 1
Cluster 2
Y04/1907
Y04/1071
Y04/1379
Y05/1504
Y05/1029, Y05/1059, Y05/1094
Y05/1033, and others (23)
Y05/2107
Y06/379
Y06/161, Y06/451
Y06/55
Y05/1849, Y05/2702
Y05/2637
Y07/1437, Y07/1477
Y07/1412
Y05/1356
Y05/1357
Y06/890, Y06/1093
Y08/2017
Y09/2272
Y09/2573
Y09/2352
Y09/2531
Y07/1645
Y05/1774
Y05/2105
Y08/1154
Y07/2125
Y08/1663
Y05/1932
Y06/360
Y06/1484
Y05/2227
Y07/400, Y07/1759
Y05/2624
Y07/2187, Y07/2227
Y07/1916
Y05/2570
Y05/862,and others (4)
Y06/305
Y05/1501, and others (4)
Y07/1662
Y06/1116
Y07/1562
Y06/3027
Y05/1388
Y07/1577, Y07/1599, Y07/2211
Y07/2354
Y08/413
Y07/2072
Y07/2274
Y09/2225, and others (4)
Y09/2019
Y09/1292, and others (10)
Y09/1653
Y09/1251
Y09/1542
Y09/1442
Y09/1660, Y09/1686
Y09/1786
Y09/1441
Y09/1412, and others (8)
Y09/2055
Y09/1346
Y09/1681
Y09/2009, and others (3)
Y09/2075
Y09/2143
Y09/1972
Y08/2018
Y07/1657, Y08/2276
Y07/1907
Y09/2581
Y04/1279
Y08/982
Y03/1644
Y03/1764
Y03/1368
Y03/1521
Y03/1523
Y03/2101
Y04/1773
Y04/759
Y03/1323, Y03/2066, Y03/1665
Y03/1446
Y03/2030
Y05/1109, and others (21)
Y05/1789
Y05/1346
Y02/1433,Y02/1565
Y03/1122
Mil-48/91 (U70936)
Washington/1964 (AF457102)
Mil-58/91 (U70943)
0.005

Figure 1


Branch length
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0.01
0.02
0.03
0.04
0.05
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HPIVi/Yamagata/2003/1122
HPIVi/Yamagata/2004/1071
HPIVi/Yamagata/2004/1907
HPIVi/Yamagata/2006/161
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HPIVi/Yamagata/2009/2272
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HPIVi/Yamagata/2009/2531
HPIVi/Yamagata/2008/2017
HPIVi/Yamagata/2005/2227
HPIVi/Yamagata/2005/862
HPIVi/Yamagata/2008/1663
HPIVi/Yamagata/2005/1029
HPIVi/Yamagata/2005/1388
Cluster 1
HPIVi/Yamagata/2003/1644
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HPIVi/Yamagata/2005/1789
HPIVi/Yamagata/2004/1279
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HPIVi/Yamagata/2007/2072
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HPIVi/Yamagata/2007/1657
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HPIVi/Yamagata/2009/2009
HPIVi/Yamagata/2009/2075
HPIVi/Yamagata/2009/2055
HPIVi/Yamagata/2009/1251
HPIVi/Yamagata/2009/1441
HPIVi/Yamagata/2009/1786
HPIVi/Yamagata/2009/2019
HPIVi/Yamagata/2009/1653
HPIVi/Yamagata/2003/1323
HPIVi/Yamagata/2003/2030
HPIVi/Yamagata/2004/1773
HPIVi/Yamagata/2003/2101
Cluster 2
Washington/1964 (AF457102)
Mil-58/91 (U70943)
1950
1960
1970
1980
1990
2000
2010
Year

Figure 2

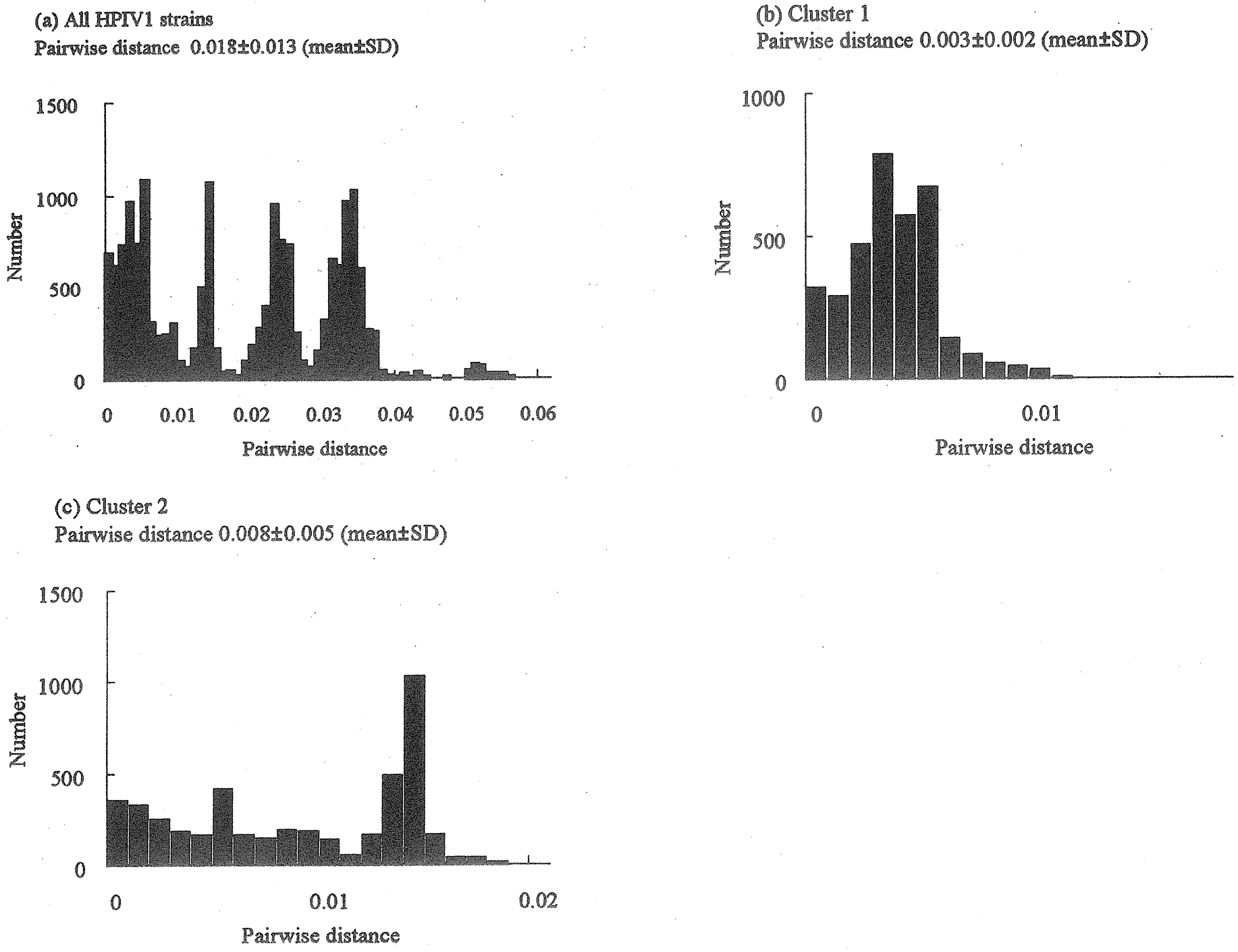
(a) All HPIV1 strains
Pairwise distance 0.018±0.013 (mean±SD)
Number
1500
1000
500
0
0
0.01
0.02
0.03
0.04
0.05
0.06
Pairwise distance
(b) Cluster 1
Pairwise distance 0.003±0.002 (mean±SD)
Number
1000
500
0
0
0.01
Pairwise distance
(c) Cluster 2
Pairwise distance 0.008±0.005 (mean±SD)
Number
1500
1000
500
0
0
0.01
0.02
Pairwise distance

Figure 3

Additional files provided with this submission:

Additional file 1: supp1.doc, 32K
http://www.virologyj.com/imedia/2102460479647382/supp1.doc

Original Article

Prevalence and Genotype of *Salmonella* Choleraesuis in Gunma Prefecture, Japan

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Abstract

We studied the prevalence of swine salmonellosis and PFGE genotype of isolates in Gunma Prefecture, Japan. Between 2005 and 2008, swine salmonellosis was confirmed in 430 of 2,707,402 (0.02%) swine at slaughterhouses. All isolates were identified as deriving from *Salmonella* Choleraesuis, biotype Choleraesuis (negative for H_2S production). We used 30 bacterial strains from 15 farms that had experienced outbreaks in 2006 and 2007. All strains were susceptible to various antibiotics such as cephems (cefotaxime), fluoroquinolones (norfloxacin and ciprofloxacin), and fosfomycin. On the other hand, all strains were resistant to tetracycline (TC), and 29 of 30 (97%) strains were resistant to streptomycin (SM). The most predominant profiles were those of SM-TC (26 strains). During *Bln* I digestion, 30 strains showed 6 profiles on PFGE as G1 to G6, and each profile was assigned into 1 of 4 clusters (I to IV). The most prevalent profile was G1 (22 strains), followed by G3 (3 strains), and G2 (2 strains). Strains showing the same antimicrobial resistance profiles (SM-TC) and the same PFGE profiles (G1) were isolated from 5 of 15 farms (A to E) during the 2006 and 2007 outbreaks. In conclusion, the prevalence of swine salmonellosis caused by SM-TC resistant-*S.* Choleraesuis biotype Choleraesuis is around 0.02%, as determined by infection rate at pig farms between 2005 and 2008 in Gunma prefecture. *S.* Choleraesuis usually causes systemic infections in swine and humans and antimicrobial treatment is necessary. The antimicrobial susceptibility of *Salmonella* in swine should be surveyed further.

Keywords: antimicrobial resistance, genotyping, pig, *Salmonella*

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บทคัดย่อ

ความชุก จีโนทัยป์ของเชื้อ *Salmonella* Choleraesuis ในจังหวัด Gunma ประเทศญี่ปุ่น

Chieko Sakano[1] Yukio Morita[1,2] Koichi Goto[1] Yoko Yokota[1] Hideyuki Annaka[1] Masahiro Fujita[1] Satoshi Kobatake[1] Taisei Ishioka[1] Toshie Hoshino[1] Sumalee Boonmar[1] Chaiwat Pulsrikarn[3] Atsuyoshi Nishina[4] Kunihisa Kozawa[1] Shigeki Yamamoto[5] Hirokazu Kimura[1,6]*

การศึกษาความชุกของโรคซัลโมเนลลาในสุกรและจีโนทัยป์ของเชื้อซัลโมเนลลาโดยวิธี PFGE ในจังหวัด Gunma ประเทศญี่ปุ่น ผลการศึกษา พบว่าระหว่างปี ค.ศ. 2005-2008 มีความชุกของโรคนี้ในสุกร ประมาณร้อยละ 0.02 คือ พบว่าสุกรจำนวน 430 ตัว (จาก 2,707,402 ตัว) ที่โรงงานฆ่าสัตว์ มีการติดเชื้อซัลโมเนลลา โดยเชื้อทั้งหมดเป็น *Salmonella* Choleraesuis ชนิดไม่สร้าง H2S ทำการทดลองโดยนำ เชื้อซัลโมเนลลาจำนวน 30 ตัวอย่าง ที่เพาะแยกจากฟาร์มสุกร 15 ฟาร์มในปีที่มีการระบาดของโรคนี้ คือปี ค.ศ. 2006-2007 มาศึกษาหาความไวของเชื้อต่อยาปฏิชีวนะ พบว่าเชื้อทั้งหมดไวต่อยาจำนวนมาก เช่น cephems (cefotaxime) quinolones (norfloxacin และ ciprofloxacin) และ fosfomycin ในทางตรงข้ามพบว่าเชื้อทั้งหมดดื้อต่อยา tetracycline (TC) และร้อยละ 97 ของเชื้อดื้อต่อยา streptomycin (SM) โดยมี แบบ(profile) การดื้อยาเป็นแบบ SM-TC (จำนวน 26 ตัวอย่าง) เมื่อนำเชื้อมาย่อยด้วยเอนไซม์ *Bln* I ในขบวนการ PFGE พบว่า สามารถแบ่งเชื้อเป็น 6 แบบ (profile) คือ แบบ G1 ถึง G6 และแบ่งย่อยได้เป็น 4 cluster (I ถึง IV) โดยแบบ G1 ประกอบด้วย เชื้อ 22 ตัว แบบ G3 ประกอบด้วย เชื้อ 3 ตัว และแบบ G2 ประกอบด้วย เชื้อ 2 ตัว เชื้อที่มีรูปแบบการดื้อยาเป็นแบบ SM-TC และมีแบบ PFGE อยู่ในกลุ่ม G1 นั้นมาจากฟาร์มสุกร 5 ฟาร์ม (ในจำนวน 15 ฟาร์ม) คือ ฟาร์ม A ถึง E ระหว่างที่มีการระบาดของโรคนี้ในปี ค.ศ. 2006-2007 จากการศึกษาสามารถสรุปได้ว่า ความชุกของโรคซัลโมเนลโลซีสในฟาร์มสุกร คิดเป็นร้อยละ 0.02 และเป็นเชื้อ *Salmonella* Choleraesuis ที่ดื้อต่อยา SM-TC ในระหว่างปี 2005-2008 ในจังหวัด Gunma ประเทศญี่ปุ่น เชื้อนี้ทำให้เกิดโรคทั้งในสุกรและคนและมีความจำเป็นต้องใช้ยาปฏิชีวนะในการรักษา ดังนั้นควรมีการศึกษาเรื่องการดื้อยาของเชื้อนี้ในสุกรต่อไป

คำสำคัญ: การดื้อต่อยาปฏิชีวนะ จีโนทัยป์ สุกร เชื้อซัลโมเนลลา

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Introduction

More than 2,500 serovars of non-typhoid *Salmonella* (NTS) have been confirmed (Callaway et al., 2008). NTS is a significant food-borne agent with worldwide distribution. In general, NTS persists by using animal intestines as reservoirs where it survives in a dormant state. However, some serovars such as Gallinarum-Pullorum, Dublin, Enteritidis, Typhimurium, and Choleraesuis can cause serious disease in domestic animals (Shivaprasad, 2000; Callaway et al., 2008; Pullinger et al., 2008). Many serovars, such as Enteritidis, Typhimurium, and Infantis, of *S. enterica* subsp. *enterica* are frequently isolated from both swine and humans. In particular, *S.* Choleraesuis is a host-adapted, facultative, intracellular pathogen that causes swine paratyphoid fever (Wilcock and Scwarts, 1992).

There are two biotypes of *S.* Choleraesuis. Choleraesuis is H_2S-negative and Kunzendorf is H_2S-positive in Triple Sugar Iron (TSI) agar (Sato, 1987). In Japan, *S.* Choleraesuis was first isolated in 1928 and swine salmonellosis caused by *S.* Choleraesuis occurred sporadically in the 1970s (Sato, 1987). In the past 10 years from 2010, swine salmonellosis caused by *S.* Cholaraesuis has increased (Asai et al., 2010).

In humans, *S.* Choleraesuis infection is responsible for salmonellosis, particularly in the elderly and in immunocompromised patients (Chiu et al., 2004). In the United State and United Kingdom,

S. Choleraesuis was sporadically isolated from humans (Barrel, 1987; Vugia et al., 2004). In Asian countries, especially in Taiwan, *S.* Choleraesuis is a significant serovar isolated from both humans and animals. In humans, it is frequently identified as the main cause of salmonellosis (Chiu et al., 2004). In Japan, the number of humans infected by *S.* Choleraesuis remains very low in spite of increased outbreaks in swine. Only a few reports regarding swine salmonellosis caused by *S.* Choleraesuis in Japan have been published. Asai et al. (2010) reported that both biotypes of *S.* Choleraesuis such as Choleraesuis and Kunzendorf exist in Japan, especially west of Japan. Biotype Choleraesuis was classified as one big cluster, while biotype Kunzendorf was divided into two big clusters according to the results of pulsed-field gel electrophoresis (PFGE) comparing biotype Choleraesuis and Kunzendorf. However, the results of field epidemiological studies were obscure. In Gunma prefecture, located in center of Japan geographically, there is no *Salmonella* pig cases in meat inspection, but we began to find the *Salmonella* cases on viscera inspection from 2005. Here, we report the field epidemiological study in Gunma prefecture.

In Japan, according to the report of the local infectious disease survey center, the National Institute of Infectious Disease (http://idsc.nih.go.jp/iasr/virus/bacteria-j.html), isolation of *S.* Choleraesuis from humans is not common and bacteria have been isolated only once each year in 2004, 2005, 2007, and 2008. In Taiwan, however, human systemic infection by *S.* Choleraesuis is common and emergence of fluoroquinolone-resistant salmonella has become a serious problem (Chiu et al., 2002). Considering such aspects, we studied the prevalence, genotyping and antimicrobial susceptibility of *Salmonella enterica* subsp. *enterica* serovar Choleraesuis isolated in Gunma Prefecture, Japan.

Materials and Methods

Samples: Between 2005 and 2008, a total of 2,707,402 swine were inspected for salmonellosis by viscera inspection at G slaughterhouse in Gunma Prefecture. When swine with suspicious *Salmonella* systemic disease were detected during viscera inspection by a veterinary meat inspector, the swine samples of liver, lung and/or lymph nodes lesions were taken for bacteriological examination.

Bacteriological examination: Swine samples of liver, lung and/or lymph nodes lesions collected in viscera inspection were stored at 4°C and analyzed within 4 hours of sampling. One to two grams of each swine sample was inoculated into 10 ml of tetrathionate broth (Oxoid, Hampshire, UK) and rappaport-vassiliadis (RV) enrichment broth (Oxoid). The broth was incubated at 42°C for 20 hours under aerobic conditions. After incubation, a loopful of broth culture was streaked across brilliant green sulpha agar (BGS agar) (Oxoid) and double modified lysine iron agars (dmLIA : own composition; pH 6.7, containing 34 g of lysine iron agar, 1.5 g of bile salts No. 3, 10 g of lactose, 10 g of sucrose, 6.76 g of sodium thiosulfate, 0.3 g of ferric ammonium citrate, 0.015 g of sodium novobiocin, and 1 liter of distilled water) and incubated at 37°C for 24 hours under aerobic conditions. One to three presumptive *Salmonella* colonies such as red color colony on BGS agar and purple color colony on dmLIA were selected and sub cultured onto triptosoy agar (Oxoid) for 24 hours at 37°C under aerobic conditions. Colonies growing on the agar were selected according to the Laboratory Guidebook of the United State Department of Agriculture (http://www.fsis.usda.gov/PDF/MLG_4_04.pdf). Each *Salmonella* isolate was serotyped by a combination of O and H reactions using commercial antiserum (Denka, Tokyo), and the resulting serotype was identified by the Kauffman-White serotyping scheme. The isolates were classified into biotypes by confirming their H_2S production capability in sulfide-indole-motility (SIM) media (Nissui, Tokyo). When the slaughtered pig has suspicious salmonellosis systemic infection and *Salmonella* were isolated from lesions using bacteriological examination, we defined as salmonellosis.

Antimicrobial susceptibility of the isolates: We examined 30 strains of antimicrobial susceptibility test. The 30 strains were *S.* Choleraesuis from 15 pig farms that harbored swine salmonellosis both 2006 and 2007. Antimicrobial susceptibility of the isolates was examined by the disk diffusion method using Mueller-Hinton agar (Oxoid) plates (Bauer et al., 1966). Twelve types of antimicrobial disks (BD, NJ, USA) were used for the tests: 10 μg of ampicillin (AMP); 25 μg of amoxicillin (AMPC); 30 μg of cefotaxime (CTX); 30 μg of kanamycin (KM); 10 μg of streptomycin (SM); 30 μg of tetracycline (TC); 30 μg of chloramphenicol (CP); 30 μg of nalidixic acid (NA); 10 μg of norfloxacin (NOR); 5 μg of ciprofloxacin (CIP); 50μg of fosfomycin (FOM); 23.75 μg of sulfamethoxazole; and 1.25 μg of trimethoprim (ST). Next, we determined isolate resistance to AMP and AMPC using 10 μg of ampicillin with 10 μg of sulbactam sodium/ampicillin sodium (ABPC/SBT) disk: BD to investigate β-lactamase production. In these tests, *Escherichia coli* ATCC25922 was used for the control. The breakpoint for the antimicrobial drugs was based on the guidelines provided by the National Committee on Clinical Laboratory Standard (2002).

Pulsed-field gel electrophoresis: PFGE was performed according to the Pulse Net standardized protocol (Ribot et al., 2006). Briefly, plugs were digested for 3 hours with 25 U of *Bln* I (Takara, Tokyo). DNA fragments were separated by 1% agarose gel electrophoresis (Takara) using a CHEF DR-III PFGE system (Bio-Rad, CA, USA). CHEF DNA size standards lambda ladder (Bio-Rad) was used as a molecular size marker.

DNA fingerprint analysis: DNA fingerprints were analyzed with Fingerprinting II Software (Bio-Rad). After an automatic band search and a band-based analysis using Dice's coefficient with 1.5% band position tolerance, cluster analysis was performed by using the un weighted pair-group method with

arithmetic averages (UPGMA) with 85% similarly index.

Results

Prevalence of swine salmonellosis: Examination revealed that 430 (0.02%) of 2,707,402 inspected swine were infected with salmonellosis. Swine salmonellosis was found in 35 (0.01%) of 662,270 swine in 2005, 198 (0.03%) of 655,203 in 2006, 115 (0.02%) of 686,613 in 2007, and 82 (0.01%) of 703,316 in 2008. Isolates from all the infected swine were identified as *S.* Choleraesuis and were classified as biotype Choleraesuis (H_2S negative). We were unable to isolate biotype Kunzendorf (H_2S positive) in this study.

Antimicrobial susceptibility of the isolates: Table 1 shows antimicrobial resistance profiles of isolated *S.* Choleraesuis. By using 12 types of antimicrobial drugs, we determined that not all strains were resistant to CTX, NOR, CIP, and FOM, whereas all strains were resistant to TC and 29 of 30 (97%) strains showed resistance to SM. The 30 strains were classified into 4 groups and a profile was established for each. The profiles were based on the resistance to the 8 antimicrobial agents. The most predominant profiles were that of SM-TC (26 strains), followed by SM-TC-NA (2 strains), AMP-AMPC-KM-SM-TC-ST (1 strain), and AMP-AMPC-KM-TC-CP-ST (1 strain). Two strains with resistance to AMP-AMPC were noted as capable of β-lactamase production as they demonstrated susceptibility to the ABPC/SBT disk.

Table 1 Antimicrobial resistance profiles of isolated S. Choleraesuis

Antimicrobial agent [a)]	No. of Strain	Isolation year	
		2006	2007
SM-TC	26	13	13
SM-TC-NA	2	1	1
AMP-AMPC-KM-SM-TC-ST	1[b)]	0	1
AMP-AMPC-KM-TC-CP-ST	1[b)]	1	0
Total	30	15	15

a) SM: streptomycin, TC: tetracycline, NA: nalidixic acid, AMP: ampicillin, AMPC: amoxicillin, KM: kanamycin, CP: chloramphenicol, and ST: sulfamethoxazole with trimethoprim. b) β-lactamase production

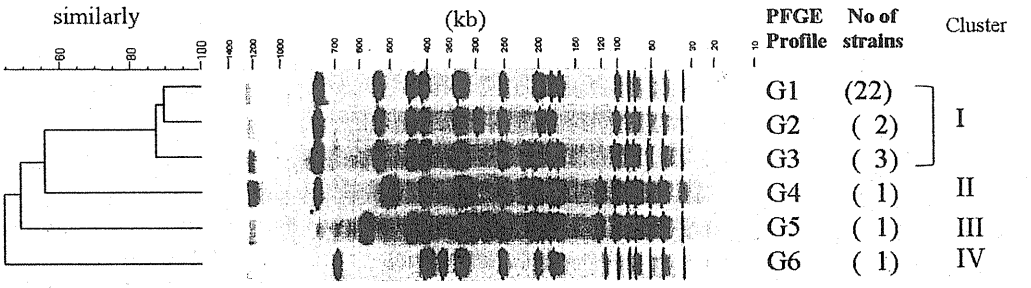

Figure 1 Diagram of PFGE profiles according to UPGMA algorithms with 85% similarly index in *S.* Choleraesuis biotype Choleraesuis by *Bln I*-digested chromosomal DNA of the 30 strains.

Table 2 Profiles of antimicrobial resistance and PFGE of S. Choleraesuis isolated in 2006 and 2007.

No.	Farm	2006		2007	
		Antimicrobial agent [a)]	PEGE profile	Antimicrobial agent [a)]	PEGE profile
1	A	SM-TC	G1	SM-TC	G1
2	B	SM-TC	G1	SM-TC	G1
3	C	SM-TC	G1	SM-TC	G1
4	D	SM-TC	G1	SM-TC	G1
5	E	SM-TC	G1	SM-TC	G1
6	F	SM-TC	G1	SM-TC	G2
7	G	SM-TC	G1	SM-TC	G3
8	H	SM-TC	G1	SM-TC	G5
9	I	SM-TC	G2	SM-TC	G6
10	J	SM-TC	G3	SM-TC	G1
11	K	SM-TC	G3	SM-TC	G1
12	L	SM-TC	G1	AMP-AMPC-KM-SM-TC-ST	G1
13	M	SM-TC-NA	G1	SM-TC	G1
14	N	SM-TC	G1	SM-TC-NA	G1
15	O	AMP-AMPC-KM-TC-CP-ST	G4	SM-TC	G1

a) SM: streptomycin, TC: tetracycline, NA: nalidixic acid, AMP: ampicillin, AMPC: amoxicillin, KM: kanamycin, CP: chloramphenicol, ST: sulfamethoxazole with trimethoprim.

Diagram of PFGE profiles: Figure 1 shows a diagram of PFGE profiles according to UPGMA algorithms of the Choleraesuis biotype by *Bln* I-digested chromosomal DNA of the 30 strains. During *Bln* I digestion, the strains showed 6 profiles (G1 to G6), each of which was assigned to 1 of 4 clusters (I to IV). Strains of G1 to G3 were assigned to cluster I and showed 85% similarity. The most predominant profile of cluster I was G1 (22 strains) followed by G3 (3 strains) and G2 (2 strains). G4 strains were assigned to cluster II, G5 to cluster III, and G6 to cluster IV; each profile having only one strain.

Relationship between isolation year, farm, result of antimicrobial resistance, and PFGE profiles: Table 2 shows the profiles in relation to antimicrobial resistance and PFGE of *S.* Choleraesuis isolated at 15 farms during the 2006 and 2007outbreaks. Strains showing the same antimicrobial resistance profiles (SM-TC) and same PFGE profiles (G1) were most predominant and the strain was isolated from 14 of 15

farms during the experiment period. In both 2006 and 2007, the same strains were isolated from 5 farms (A to E). Strains isolated from 6 farms (F to K) had the same antimicrobial resistance profile (SM-TC), but different PFGE profiles in each year. In contrast, 4 farms (L to O) showed different antimicrobial resistance and PFGE profiles in each year. There is no relationship between antimicrobial resistance and PFGE profiles in our study.

Discussion

It is reported that swine salmonellosis caused by *S. enterica* subsp. *enterica* serovar Choleraesuis has increased in Japan (Asai et al., 2010). In Gunma prefecture, we first detected swine salmonellosis during meat inspection in 2005. Since the first case, the disease has been sporadically observed between 2005 and 2008, and 0.02% (430/2,707,402) of swine were infected salmonellosis caused by *S.* Choleraesuis biotype Choleraesuis (H_2S negative) strains. Despite the widespread proliferation, no human cases or incidents of food poisoning caused by the strains have been observed to date in the prefecture.

With regard to the antimicrobial susceptibility of the isolates of 2006 and 2007, all examined strains were susceptible to various antibiotics such as cephems (CTX), fluoroquinolones (NOR and CIP), and FOM, while all strains showed resistance to TC, and 29 of 30 (97%) strains were resistant to SM. Asai et al.(2010) showed that various *S.* Choleraesuis strains isolated from 2001 to 2005 in Japan were resistance to dihydrostreptomycin (100%), oxytetracycline (69.2%), trimethoprim (40.4%), and AMP (34.6%). Although the antimicrobials examined by Asai et al. (2010) were slightly different to those of ours, most *S.* Choleraesuis isolated in Japan may show resistance to SM and TC. As a possible reason, many types of antibiotics such as penicillin, streptomycin, tetracycline, and methoprims are frequently used in Japan for treating bacterial infections in swine. Multidrug-resistant isolates, including those with fluoroquinolone and cephalosporin resistance, were found in *S.* Choleraesuis in Taiwan and biotype Kunzendorf (H_2S positive) was predominant in these countries (Chiu et al., 2004; Chang et al., 2005; Kulwichit et al., 2007). However, our strains and the previous report by Asai et al. (2010) indicate that no strains exist with resistance to fluoroquinolones (NOR, CIP, and enrofloxacin) or cephems (CTX and cefazolin). Swine-spread *S.* Choleraesuis strains in Japan are different from those of Taiwan and Thailand. However, 2 strains in the present study demonstrated multidrug resistance (AMP-AMPC-KM-SM-TC-ST and AMP-AMPC-KM-TC-CP-ST).

In diagram of PFGE profiles by using *Bln* I enzyme, examined 30 strains were divided to 6 profiles (G1 to G6) and 4 clusters (I to IV). The most predominant profile were G1 (22 strains), followed by G3 (3 strains) and G2 (2 strains), and G1 to G3 belong to cluster I. Our study shows that genetically close *S.* Choleraesuis were isolated from the infected swine in Gunma Prefecture.

When swine salmonellosis caused by *S.* Choleraesuis was detected, a veterinarian must notify the local government of the infected animals as stipulated by the Act on Domestic Animal Infectious Diseases Control of 1951, Japan. Infected animals are then treated and the animal quarters are thoroughly disinfected. However, a complete elimination of the *Salmonella* might be difficult, because of strains with the same profiles of antimicrobial susceptibility and because PFGE were isolated from the infected swine from 5 of the 15 farms in the 2006 and 2007 outbreaks.

In Japan, we usually use Salmonella Shigella agar (SS agar), deoxycholate hydrogen sulfide lactose agar (DHL agar), or mannitol lysine crystal violet brilliant green agar (MLCB agar) to isolate *Salmonella* from samples. These selective agars are able to isolate H_2S-producing *Salmonella* colonies. However, we may not be able to isolate non-H_2S-producing *Salmonella* like *S.* Choleraesuis biotype Choleraesuis. Thus, we should use another type of selective agar, such as BGS agar, Xylose lysine deoxycholate agar (XLD agar), or dmLIA which target lysine decarboxylase, or chromogenic agar.

S. Choleraesuis usually causes systemic infections in swine and humans and antimicrobial treatment is necessary. In Japan, human cases caused by *S.* Choleraesuis are very rare. However, in swine, salmonellosis occurred in swine that is usually caused in present times by *S.* Choleraesuis biotype Choleraesuis (H_2S negative), and has spread to many swine farms in Gunma prefecture, Japan. In this study, isolated *S.* Choleraesuis were not resistant to fluoroquinilones. However, in Taiwan, *S.* Choleraesuis isolated from swine and humans were resistant to fluoroquinolones and other antibiotics (Chang et al., 2005; Kulwichit et al., 2007). The antimicrobial susceptibility of *Salmonella* in swine should be surveyed further, and the selective method for isolating *Salmonella* should be reconstructed in Japan.

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Saffold Cardiovirus Infection in Children Associated With Respiratory Disease and Its Similarity to Coxsackievirus Infection

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Background: Saffold virus (SAFV) is a newly discovered virus belonging to the genus *Cardiovirus* of the family *Picornaviridae*. Using molecular techniques, SAFV has been detected, although infrequently, in the stools of both healthy and diarrheic children and in respiratory specimens collected from children with respiratory disease. The epidemiology and pathogenicity of SAFV remain unclear.

Methods: Between July 2009 and October 2010, nasopharyngeal specimens were collected from children with acute respiratory infections. The collected samples were used to isolate respiratory viruses, including coxsackievirus, by cell culture and were tested for SAFV by reverse transcription-polymerase chain reaction.

Results: SAFV genotype 2 (SAFV2) was detected in 54 (3.5%) of the 1525 children tested. SAFV2 detections showed an epidemic pattern for a 4-month period with a peak in October 2009. The median age of the SAFV2-positive children was 4 years (range: 7 months–16 years). Among the 35 SAFV2-positive children, excluding cases of viral coinfection, 13 (37.1%) had pharyngitis, 12 (34.3%) had tonsillitis, and 8 (22.8%) had herpangina. Bronchitis and gastroenteritis were detected in 1 case each. Fever (temperature, >38°C) was noted in 33 (94.3%) cases. The median duration of fever was 2 days (range: 1–3 days). Diarrhea was observed in 7 (20.0%) children, but watery and frequent diarrhea was not common. The age distribution and clinical diagnoses associated with SAFV2 infections were similar to those observed with coxsackievirus B4 infections, which detections showed an epidemic pattern during the study period.

Conclusion: SAFV2 is a cause of upper respiratory tract illness that exhibits a pathogenicity similar to that of coxsackievirus B.

Key Words: saffold virus, cardiovirus, enterovirus, coxsackievirus, herpangina

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Saffold virus (SAFV), a human cardiovirus, was recovered from a stool sample of an infant with a fever of unknown origin in 2007.[1] The newly identified virus was classified under the genus *Cardiovirus*, family *Picornaviridae*. Subsequent studies have revealed the existence of multiple genotypes encoding highly divergent VP1 protein (approximately 60%–80% amino acid identity) and have identified at least 8 genotypes (SAFV1 to SAFV8).[2–4] Whether the 8 SAFV genotypes are serologically distinct has not yet been determined. Although SAFV has been difficult to propagate in cell culture, SAFV2 and SAFV3 have recently been cultured and their seroprevalence reported.[5,6] The seroprevalence data revealed that SAFV3 infection occurs early in life and that >90% of children aged >2 years possess antibodies to SAFV3.[5] Additionally, 91% of adults carry antibodies to SAFV2.[6] These data show that SAFV infection is widespread in human beings. However, as SAFV has been detected infrequently, its pathogenicity and epidemiology remain unclear.

Using molecular techniques, SAFV has been detected in stool samples from healthy children and from children with gastroenteritis, with coinfection with enteric viruses (eg, rotavirus and norovirus) detected in many cases.[2,3,7,8] Whether SAFV is responsible for gastroenteritis remains an open question. In addition, there have been reports of SAFV detection in respiratory samples taken from children with respiratory disease.[9,10] We have also previously reported the detection of SAFV2 in nasopharyngeal swab specimens taken from 9 children with exudative tonsillitis.[11] Therefore, it is possible that SAFV is associated with respiratory disease rather than gastroenteritis. It is possible that SAFV is transmitted by both fecal-oral and respiratory routes, similar to other viruses belonging to the family *Picornaviridae*, such as enteroviruses.[12]

To clarify the etiology and epidemiology of acute respiratory infections (ARIs), we have collected respiratory specimens from patients with ARI annually since 2001 and have isolated a number of respiratory viruses, including enteroviruses, by cell culture using multiple cell lines.[13–16] In this study, we used respiratory specimens obtained between 2009 and 2010, attempted to detect SAFV by reverse transcription (RT)-polymerase chain reaction (PCR), and succeeded in the detection of an ARI-associated outbreak of SAFV2 for the first time. In this study, we report the clinical characteristics of SAFV2 and discuss its similarity to coxsackievirus A (CoxA) and coxsackievirus B (CoxB).

MATERIALS AND METHODS

Respiratory Specimens and Virus Isolation

Between July 2009 and October 2010, 1525 nasopharyngeal swab specimens were obtained from patients <18 years with ARI at the Yamanobe Pediatric Clinic. The collection was done in collaboration with the local health authority of the Yamagata Prefecture for the surveillance of viral diseases in Japan. The specimens were transported at 4°C to the Department of Microbiology, Yamagata Prefectural Institute of Public Health, and were

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inoculated into 6 different cell lines: human embryonic lung fibroblast, human laryngeal carcinoma (HEp-2), Vero E6, Madin-Darby canine kidney, rhabdomyosarcoma (RD-18S), and green monkey kidney. All cell lines were prepared in the wells of a 96-well microplate (Greiner Bio-One, Frickenhausen, Germany).[13,15] The remainder of each specimen was stored at −80°C until application to RT-PCR assay. When a suspected cytopathic effect of enterovirus was observed, viral identification and the determination of serotype were performed by neutralization test, using serotype-specific antisera as described previously.[13]

RT-PCR and Sequencing for SAFV

Viral RNA was extracted from 200 μL of specimen using a High Pure Viral RNA kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Reverse transcription and subsequent amplification of the VP1 coding region of SAFV by nested PCR were performed as previously described.[11] Amplicons were sequenced and the obtained sequences were used for phylogenetic analysis. Genotype of each amplicon was determined by a phylogenetic tree that was constructed using the neighbor-joining methods as previously described.[11]

Clinical Data

The clinical characteristics of children testing positive for SAFV in their respiratory specimens were obtained from their medical records. The case definitions of pharyngitis, herpangina, and tonsillitis were given as follows, referring to the textbooks.[17,18] Herpangina was defined by papular, vesicular, and ulcerative lesions on the soft palate, uvula, or another parts of the oropharynx. Tonsillitis was defined by red, swollen tonsils with white exudate. Pharyngitis was defined by a red pharynx and an accompanying lack of any vesicles or tonsillitis.

Statistical Analyses

Statistical analysis was performed using StatView-J 4.02. Categorical variables between groups were compared with the χ^2 test. For continuous variables, all comparisons were based on the nonparametric Mann-Whitney U test. A $P < 0.05$ was regarded as statistically significant.

RESULTS

Detection of Respiratory Viruses by Cell Culture

Between July 2009 and October 2010, respiratory viruses were isolated in 695 (45.6%) of the 1525 children with ARI. In 436 children, viruses other than picornaviruses, such as influenza virus, parainfluenza virus, respiratory syncytial virus, human metapneumovirus, and adenovirus, were isolated. A total of 263 strains belonging to the family *Picornaviridae* were isolated in 259 (17.0%) children. Rhinovirus, CoxB4, and CoxA4 were isolated most frequently (Table 1).

SAFV Detection by RT-PCR and Monthly Distribution

RT-PCR for SAFV was positive in 54 (3.5%) respiratory specimens. Sequencing and phylogenetic analysis revealed that all 54 strains were SAFV2. In all, 9 strains have already been reported in our previous article.[11] Dual infections were detected in 18 (33.3%) of the SAFV2-positive children: rhinovirus in 5 children; parainfluenza virus type 2 in 2 children; cytomegalovirus in 2 children; and CoxA2, CoxA3, CoxA4, CoxB4, influenza A virus, parainfluenza virus type 1, parainfluenza virus type 3, human metapneumovirus, and herpes simplex virus in 1 child each. The monthly distribution of SAFV2 is shown in Figure 1. During the study period, epidemic patterns, characterized by peaks in numbers of isolates followed by peaks with few isolates, were also observed for CoxA4 and CoxB4. Monthly distributions of CoxA4 and CoxB4 are also shown in Figure 1. SAFV infection peaked in October 2009 with a detection rate of 17.2% (22/128 specimens). The highest isolation rates for CoxA4 and CoxB4 were 18.5% (15/81 specimens) in July 2010 and 24.6% (16/65 specimens) in August 2010, respectively.

TABLE 1. Viruses Belonging to the Family *Picornaviridae* Isolated by Cell Culture Between July 2009 and October 2010

Virus	No. (%) Positive Children*
CoxA2	11 (0.7)
CoxA3	3 (0.2)
CoxA4	40 (2.6)
CoxA6	3 (0.2)
CoxA9	2 (0.1)
CoxA10	14 (0.9)
CoxA16	14 (0.9)
CoxB2	3 (0.2)
CoxB3	24 (1.6)
CoxB4	61 (4.0)
Echovirus 11	17 (1.1)
Enterovirus 71	10 (0.7)
Rhinovirus	61 (4.0)

*Total number of children tested was 1525.
Cox indicates coxsackievirus.

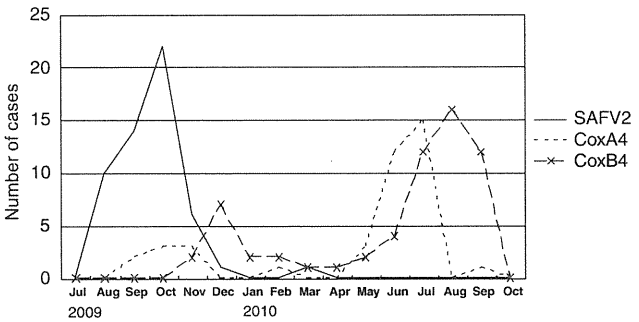

FIGURE 1. Monthly distribution of SAFV2, CoxA4, and CoxB4 detected in Yamagata, Japan, between July 2009 and October 2010.

TABLE 2. Positive Rates of SAFV2, CoxA4, and CoxB4 by Age Group

Age Group (yr)	Total Tested	No. (%) Children Infected With		
		SAFV2	CoxA4	CoxB4
0–1	472	8 (1.7)	8 (1.7)	11 (2.3)
2–4	454	23 (5.1)	27 (5.9)	25 (5.5)
5–11	469	20 (4.3)	5 (1.1)	23 (4.9)
12–17	130	3 (2.3)	0	2 (1.5)
Total	1525	54 (3.5)	40 (2.6)	61 (4.0)

SAFV indicates saffold virus; Cox, coxsackievirus.

Frequency of Infection of SAFV2, CoxA4, and CoxB4 Among Different Age Groups of Children

The positive rates of SAFV2, CoxA4, and CoxB4 by age group are shown in Table 2. The age distribution of SAFV2-infected children is shown in Figure, Supplemental Digital Content 1, http://links.lww.com/INF/A787. The positive rates of SAFV2 were similar to those of CoxB4 across all age groups. The mean

TABLE 3. Clinical Diagnoses Due to SAFV2, CoxA4, and CoxB4

Diagnosis	SAFV2 (n = 35)	CoxA4 (n = 29)	CoxB4 (n = 47)
Pharyngitis	13 (37.1)	8 (27.6)	17 (36.2)
Herpangina*	8 (22.8)	15 (51.7)	14 (29.8)
Tonsillitis	12 (34.3)	6 (20.7)	16 (34.0)
Bronchitis	1 (2.9)	0	0
Gastroenteritis	1 (2.9)	0	0

Data are number (%) of children.

*P value is 0.0166 for comparison of children positive for SAFV2 and children positive for CoxA4.

SAFV indicates saffold virus; Cox, coxsackievirus.

ages for children testing positive for SAFV2, CoxA4, and CoxB4 were 4.7 ± 3.4 years (range: 7 months–16 years; median: 4 years), 3.0 ± 1.7 years (range: 9 months–7 years; median: 3 years), and 4.5 ± 3.3 years (range: 3 months–12 years; median: 3 years), respectively. The frequency of virus detection in younger children (0–4 years) was 57.4% (31 of 54) for SAFV2, 87.5% (35 of 40) for CoxA4, and 59.0% (36 of 61) for CoxB4. SAFV2 was found significantly more frequently in children ≥5 years as compared with CoxA4 (23 of 54 [42.6%] vs. 5 of 40 [12.5%]; $P = 0.0016$).

Clinical Features Associated With SFAV2 Detection

The clinical features of children testing positive for SAFV2 are presented in Table, Supplemental Digital Content 2, http://links.lww.com/INF/A788, which illustrates the characteristics, diagnosis, and symptoms of all children, age group of 0–4 years, and age group of 5–17 years). Among the 54 SAFV2-positive cases, 18 were excluded because of coinfection with another respiratory virus. One case was not included in this analysis because of the appearance of Kawasaki disease. Of the remaining 35 cases, 20 (57%) were females. Upper respiratory tract infections such as pharyngitis, herpangina, and tonsillitis were the leading diagnoses, whereas lower respiratory tract infections such as bronchitis occurred in only 1 case (Table 3). Fever (temperature, >38°C), the only 1 symptom observed in more than 50% of cases, was noted in 33 (94.3%) cases. The median temperature was 39.0°C (range: 37.6–40.2), and the median duration of fever was 2 days (range: 1–3 days). Cough, rhinorrhea, and diarrhea were present in 14 (40.0%), 11 (31.4%), and 7 (20.0%) cases, respectively. Complaints of sore throat, diarrhea, headache, and muscle aches were more frequently raised by children in the 5 to 17 year age group than those aged 0 to 4 years. However, there were no significant differences in the clinical characteristics observed between younger children aged 0 to 4 years and older children aged 5 to 17 years. Aseptic meningitis was suspected in a 16-year-old boy, though his cerebrospinal fluid was not examined. He exhibited high fever, strong headache, and a stiff neck. These symptoms were self-limiting and disappeared within 3 days.

We next compared the clinical characteristics among children diagnosed with pharyngitis, herpangina, and tonsillitis. There were no significant differences in median age, median temperature, median duration of fever, or the frequency of clinical symptoms (data not shown).

We identified a case of household infection from the index case, a 7-year-old girl, to her mother (age, 32 years). After the occurrence of high fever in the index case on September 11, her mother developed fever on September 16. Fortunately, as she visited the author's clinic (Yamanobe Pediatric Clinic), we were able to collect respiratory specimens and subsequently detected SAFV2 in those specimens. Both the index case and her mother were diagnosed with pharyngitis and a high fever, which persisted for 2 days.

Comparison of Clinical Diagnoses of Children With SAFV2, CoxA4, and CoxB4 Infection

After excluding the cases of coinfection with another respiratory virus, the clinical diagnoses of children with CoxA4 and CoxB4 monoinfections were investigated, and compared with those of children with SAFV2 monoinfection (Table 3). All children with CoxA4 and CoxB4 infection were diagnosed with upper respiratory tract infections, such as pharyngitis, herpangina, and tonsillitis. Although there were no significant differences in the association of SAFV2 infection and CoxB4 infection with the frequency of a particular clinical diagnosis, SAFV2 infection was less likely to be associated with herpangina than infection with CoxA4 ($P = 0.0166$).

DISCUSSION

We found 3.5% of the SAFV2-positive children by RT-PCR of respiratory specimens collected from children diagnosed with ARI (54/1525 specimens). In addition, we also detected an epidemic of SAFV. To the best of our knowledge, this is the first report of an SAFV epidemic. These findings indicate that SAFV2 pathogenicity is associated with respiratory infection.

In previous large studies,[2,7,8] SAFV was detected in fecal samples from children with gastroenteritis at frequencies of 0.5% (3/631), 1.2% (6/498), and 3.2% (12/373). Among the 21 SAFV strains detected, 15 belonged to genotype 1, 4 belonged to genotype 2, and 2 belonged to genotype 3. Coinfection with enteric viruses (eg, rotavirus, norovirus, and sapovirus) was detected in 17 of 21 SAFV-positive children (81%). Therefore, it remains unclear whether SAFV is responsible for gastroenteritis. SAFV genotypes 3, 4, 5, and 7 were also found in the stool of Pakistani and Afghani children with nonpolio acute flaccid paralysis at a collective frequency of 9% (5/57).[4] Further studies are necessary to clarify the role of SAFV genotypes in respiratory infection, as different enterovirus serotypes and genotypes have been associated with different symptoms.[12]

Analyses of detection month, age group, and clinical diagnosis showed similarities between SAFV2 and coxsackievirus infection. In this study, SAFV2 was detected between August and November. This epidemic pattern is similar to the pattern exhibited by enteroviruses, with summer–fall seasonality and a 3- to 4-month activity period.[15,16,19] Enterovirus surveillance data from the United States have revealed that long-term circulation patterns for individual serotypes of enterovirus varied and that the increased activity of each serotype occurred at irregular intervals.[19] From 2004 to 2010, in Yamagata, Japan, increased activity of CoxA4 was observed in 2004, 2006, and 2010. Increased CoxB4 activity was observed in 2006 and 2010. Just as coxsackievirus epidemics are not detected annually, SAFV epidemics are not likely to occur every summer–fall season, since only 1 strain of SAFV2 was detected in 2010 in Yamagata. However, further studies are needed to clarify the circulation patterns of SAFV.

The group of children aged 2 to 4 years was most commonly associated with SAFV2 infection, in agreement with what has been observed in infections of CoxA4 and CoxB4. In recent studies, of 46 reported SAFV-positive specimens, 41 (89.1%) have been from children <5 years of age.[1–4,7–10] In this study, of the 54 SAFV2-positive children, 23 (42.6%) were ≥5 years. The difference could be explained by differences in study design (eg, fecal or respiratory specimens or age of study population). Most children become infected with SAFV during the first 5 years of life, but a proportion of the children ≥5 years of age likely remain susceptible to SAFV

infection. Additional serological and epidemiological data are needed to clarify how the age of infection might be influenced by epidemic periodicity.

Our study showed that SAFV2 infection in children is associated with upper respiratory tract infections and accompanied by a sudden onset of high fever, which persists for 1 to 2 days. Pharyngitis, herpangina, and tonsillitis are common diagnoses associated with SAFV2 infection. Diarrhea was observed in 7 (20.0%) children, but watery and frequent diarrhea was not common. Therefore, it could not be concluded whether SAFV2 is associated with gastroenteritis. In general, severe illnesses such as meningitis caused by enteroviruses are observed most frequently in children aged 5 to 14 years.[12,20] In the present study, there were no significant differences detected regarding the clinical symptoms and final diagnoses of children aged 0 to 4 years and of those aged 5 to 16 years, although we did detect a suspected case of meningitis in a 16-year-old boy. Furthermore, an adult household-infection case showed similar symptoms to a pediatric case 5 days later.

The clinical diagnoses observed with SAFV2 infection were similar to that of CoxB4, but CoxA4 was more frequently associated with herpangina than SAFV2 was. In a study of the correlation between human enterovirus (HEV) species and clinical manifestation, HEV-A (including CoxA4) caused significantly more herpangina than did HEV-B (including CoxB1–6), and HEV-B caused significantly more pharyngitis and respiratory tract infections than did HEV-A.[21] Considering these findings, it is possible that the pathogenicity of SAFV2 is similar to that of CoxB-based illnesses. Further studies are required to investigate whether SAFV can cause severe illnesses such as myocarditis or encephalitis.

Our study has the limitations of methodology. Molecular methods were only used to detect SAFV, therefore, coinfection with SAFV2 and other viruses that were not detected by culture may have been present in some cases. However, because we could detect an epidemic of SAFV2 by using respiratory samples, we conclude that SAFV2 is one of the causative agents of ARI detected in respiratory samples of infected children. To date, at least 8 SAFV genotypes have been identified. Additional epidemiologic studies are needed to ascertain the epidemiological and clinical characteristics of each genotype of SAFV.

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LETTER TO THE EDITOR

The impact of Saffold cardiovirus in patients with acute respiratory infections in Yamagata, Japan

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To the Editor,

Saffold cardiovirus (SAFV) was detected in 1982 in a child with fever of unknown origin, and is classified into the family Picornaviridae and genus Cardiovirus [1]. Recent studies have suggested that SAFV may be associated with various diseases such as the common cold [2], tonsillitis [3], and gastroenteritis [4,5]. However, the epidemiology and pathogenicity of SAFV are not well understood. Thus, to assess the impact of SAFV infections on acute respiratory infections (ARI), we investigated for the presence of SAFV in Japanese patients with ARI in 2008.

Between January and December 2008, nasopharyngeal swabs from 423 patients with ARI were tested for SAFV (Table I). Using cell culture methods and reverse transcriptase polymerase chain reaction (RT-PCR), these specimens were found to be negative for influenza virus (subtypes A and B), parainfluenza virus (subtypes 1, 2, and 3), adenovirus, respiratory syncytial virus, human metapneumovirus, and human rhinovirus [6]. The parents of all subjects gave informed consent for the donation of the nasopharyngeal samples used in this analysis. All patients lived in Yamagata Prefecture and were aged between 0 and 41 y (mean ± standard deviation 5.6 ± 6.1 y). We attempted to detect SAFV using a nested RT-PCR. We extracted RNA from the samples and amplified the VP1 coding region of SAFV by nested RT-PCR as previously described [3]. The primer sequences were as follows: 5′-HAA RCA RGR YTG GAR YTT YNT NAT GTT-3′ (primer 315F) and 5′-DGG BCK DGG RCA RWA VAC YCT CAT-3′ (primer 738R) as outer primers, and 5′-AAR CAR GRY TGG ARY TTY DTH ATG TTY TC-3′ (primer 316F) and 5′-RTT RKK RAA RTY NGM RDA NCY RTT RAA CCA-3′ (primer 621R) as inner primers. To prevent carry-over contamination of the nested PCR, we took general precautions as previously described [7].

Table I. Monthly detection of Saffold cardiovirus.

Month	No. positive/No. tested	Detected %
January	2/67	3.0
February	0/58	0
March	2/32	6.3
April	0/51	0
May	1/41	2.4
June	1/36	2.8
July	0/46	0
August	1/19	5.3
September	0/11	0
October	0/16	0
November	2/29	6.9
December	0/17	0

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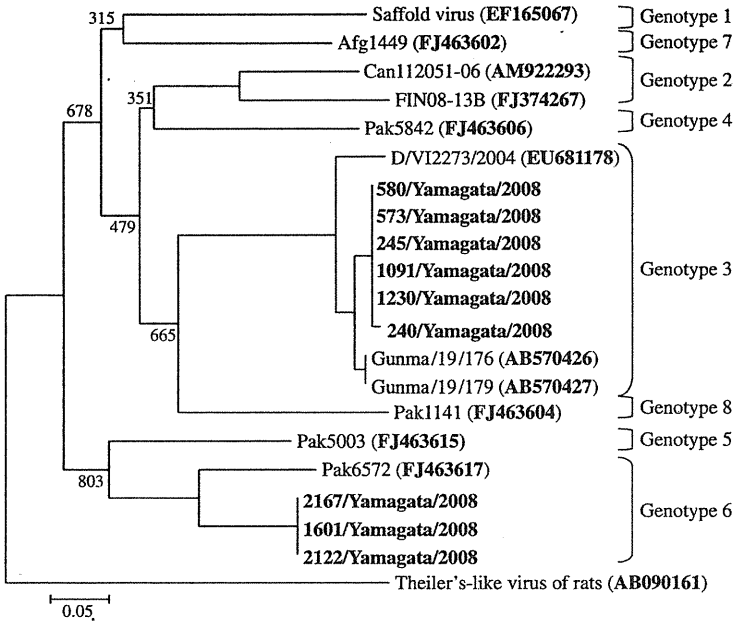

Figure 1. A phylogenetic tree of Saffold cardiovirus based on the VP1 coding region (327 nt). Numbers in parentheses are the GenBank accession numbers.

Of the 423 specimens, 9 (2.1%) tested positive for SAFV by PCR. No seasonal prevalence of SAFV was found (Table I). The sex distribution of patients was 6 male (66.7%) and 3 female (33.3%) (Table II). SAFV-positive cases were aged from 1 to 11 y (5.4 ± 2.7 y). Clinical findings in patients with SAFV included upper respiratory illness, tonsillitis and herpangina (Table I).

Next, we performed phylogenetic analysis based on the amplified region by RT-PCR. Evolutionary distances were estimated using Kimura's 2-parameter method, and phylogenetic trees were constructed using the neighbor-joining method. The reliability of each tree was estimated using 1000 bootstrap replications [8]. As a result, 6 strains were SAFV genotype 3 (SAFV3) and 3 strains were genotype 6 (SAFV6) in the phylogenetic tree (Figure 1). Nucleotide identities among the present strains were 99.4–100% among SAFV3 and 100% among SAFV6. During the investigation period the predominant genotype was SAFV3 prior to June, while after June it was SAFV6. To the best of our knowledge, this is the first report of the detection of SAFV6 in patients with ARI from the Asian areas.

Although a causal relationship still needs to be demonstrated by including a control group of healthy persons, the detection of SAFV in respiratory tract specimens from patients with ARI suggests that this virus might be associated with respiratory illness. Since this study used samples from ARI patients who tested negative only for other respiratory viruses and who were not tested for any further viruses, dual infection cannot be excluded. In addition, we cannot exclude the possibility that SAFV is present asymptomatically in humans, because samples from healthy persons

Table II. Summary of patient data and Saffold cardiovirus genotype.

Sampling date	Sex	Age (y)	Clinical diagnosis	Genotype	GenBank accession No.
29 January 2008	M	6	Acute upper respiratory inflammation and tonsillitis	3	**AB614360**
29 January	F	5	Acute upper respiratory inflammation	3	**AB614361**
3 March	F	5	Acute upper respiratory inflammation	3	**AB614362**
4 March	M	5	Acute upper respiratory inflammation	3	**AB614363**
26 May	M	3	Tonsillitis	3	**AB614364**
12 June	M	1	Herpangina	3	**AB614365**
5 August	M	11	Acute upper respiratory inflammation	6	**AB614366**
18 November	M	7	Acute upper respiratory inflammation	6	**AB614367**
20 November	F	6	Acute upper respiratory inflammation	6	**AB614368**

were not tested. Based on these facts and our results, SAFV could be relevant to acute respiratory infection across all seasons in Japan. However, a more detailed analysis, including a serological survey, is warranted to examine the exact role of SAFV in human disease.

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