Prevalence and genetic characteristics of Saffold cardiovirus in China from 2009 to 2012

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The epidemiology and clinical features of the Saffold cardiovirus (SAFV) remain ambiguous. The present study was designed to systematically and intensively investigate the epidemiological features of SAFV in pediatric patients in China. Three cohorts of pediatric patients were recruited from 2009 to 2012. Cohort 1 comprised patients with acute respiratory tract infections. Cohort 2 comprised patients with diarrhea. Cohort 3 comprised hand, foot, and mouth disease (HFMD) patients. A total of 115 patients (1.6%) among 6,052 (17/1,647, 12/2,013, and 86/2,392 in cohorts 1, 2, and 3, respectively) were SAFV-positive. The samples from 82 SAFV-positive patients were successfully sequenced, and four genotypes were identified: 8 SAFV-1, 41 SAFV-2, 29 SAFV-3, and 4 SAFV-6. A significantly higher detection rate was found in the HFMD patients than in other two cohorts (both P < 0.001). A higher frequency of severe clinical outcome and nervous system manifestation were also observed in the SAFV-positive HFMD patients. Additionally, 6 (3.5%) cerebrospinal fluid and 7 (2.2%) serum samples from the HFMD-associated encephalitis patients were SAFV-positive. Based on the VP1 sequences, all four genotypes displayed distinct geographical clustering. SAFV infection might be associated with a wide clinical spectrum and contribute to HFMD.

Picornaviruses (family Picornaviridae) are small non-enveloped viruses with a single-stranded positive-sense RNA genome that encodes a single polyprotein. Picornaviruses can cause numerous symptoms in animals, including respiratory, cardiac, hepatic, neurological, mucocutaneous, and systemic diseases of varying severities. The International Committee on Taxonomy of Viruses has recently classified the Picornaviridae family into 26 genera (including 46 species) based on genetic distance, six of which potentially infect humans (Enterovirus, Hepatovirus, Parechovirus, Kobuvirus, Cosavirus, and Cardiovirus) (www.picornaviridae.com). In the Cardiovirus genus, two distinct species, namely, Theilovirus and Encephalomyocarditis virus (EMCV), were defined based on phylogenetic analysis. Cardioviruses can cause serious diseases such as myocarditis, diabetes, central nervous system conditions, and multiple sclerosis–like disease, which are mostly reported in animals1–4. Prior to 2007, only the Vilyuisk virus, a virus related to Theiler’s murine encephalomyelitis virus (TMEV), was associated with geographically restricted encephalitis-like illness in humans5–7.

Mounting evidence has recently been collected for the existence of a new human cardiovirus. In 2007, through DNase sequence-independent single-primer amplification, a previously undescribed cardiovirus was discovered from a stool sample collected from an 8-month-old child with fever of unknown origin8. Subsequent full-length viral genome sequencing and phylogenetic analysis showed that this new virus was a human cardiovirus and was closely related to but distinct from TMEV and EMCV. This virus was provisionally named Saffold virus (SAFV) and was classified into the Cardiovirus genus of the Picornaviridae family.

Since the discovery of SAFV, there has been an inspired interest in exploring the potential associations of SAFV with human diseases, particularly on its involvement in pediatric respiratory and gastrointestinal tract infection. Several studies have detected the SAFV RNA in stool samples from children with gastroenteritis in Brazil, Germany, Thailand, Denmark, USA, Malaysia, and China as well as in the respiratory samples from children with influenza-like illnesses in Canada, Japan, and China8–19. SAFV has also been found in stools from South Asian children who either had non-polio acute flaccid paralysis (AFP) or were asymptomatic20. High sero-prevalences of SAFV-2 and SAFV-3 in children from different continents indicated that SAFVs are commonly exposed viruses in young children19,21–24. SAFV was recently found in the cerebrospinal fluid (CSF), blood, and

OPEN

SUBJECT AREAS:
VIRAL INFECTION
NEUROLOGICAL MANIFESTATIONS
Epidemiology

Received
14 September 2014
Accepted
8 December 2014
Published
9 January 2015

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myocardium of a previously healthy child who experienced sudden death, suggesting that the virus might cause serious invasive infection in children. All these aforementioned studies provided evidence that SAFV might have diverse pathogenicity that targets different tissues. However, given the insufficient systematic data, the epidemiological and genetic characteristics of SAFV infection remain ambiguous. The present study was designed to systematically and intensively investigate the epidemiological features of SAFV in pediatric patients in China.

**Methods**

**Patient recruitment and sample collection.** Three cohorts of pediatric patients were recruited from March 2009 to December 2012 in Chongqing Children’s Hospital, Chongqing, China. Cohort 1 comprised hospitalized patients with acute respiratory tract infection (ARTI), which was determined based on cough, rhinorrhea, dyspnea, and/or acute fever. For recruited patients, nasopharyngeal aspirates (NPA) samples were collected upon hospital admission. Cohort 2 comprised outpatients with diarrhea, who had three loose stools in the previous 24 hours. Patients who had confirmed inflammatory bowel disease, celiac disease, cystic fibrosis, food intolerance, or patients who had any apparent clinical respiratory signs or symptoms were excluded. Stool samples were collected from the recruited patients. Cohort 3 consisted of patients who were diagnosed with hand, foot, and mouth disease (HFMD), according to the guidelines released by the Ministry of Health of the People’s Republic of China (http://www.moh.gov.cn/publicfiles/business/htmlfiles/mohyyz/s3586/201004/46884.htm). The outpatients were generally mild, with fever, conjunctivitis, and/or alimentary tract infection. For all HFMD patients, stool samples were collected, whereas for some cases, stool, throat swab, and serum samples were collected. In addition, CSF was additionally collected for some cases diagnosed with encephalitis. Healthy children who were recruited from the community during the same study period were used as controls, from whom stool, throat swab, and serum samples were also collected. None of the recruited healthy children exhibited ARTI, diarrhea, or HFMD-related symptoms during the stool, throat swab, and serum samples were also collected. None of the recruited

| Table 1 | The detection and genotyping of SAFV in three cohorts of patients |
|---------|---------------------------------------------------------------|
| Cohort 1 (n = 1647) | Cohort 2 (n = 2013) | Subtotal | Mild HFMD (n = 1525) | Severe HFMD (n = 867) | Total | P value |
| Age, months (median, range) | 9 (1–172) | 10 (1–163) | 24 (1–168) | 24 (1–156) | 24 (2–168) | 12 (1–172) | <0.001a |
| Sex, male (%) | 1096 (66.5) | 1252 (62.2) | 1462 (61.2) | 909 (59.6) | 553 (63.8) | 3810 (62.9) | 0.044b |
| Prevalence of SAFVs | 17 (1.0) | 12 (0.6) | 86 (3.6) | 40 (2.6) | 46 (5.3) | 115 (1.9) | <0.001b |
| No. of patients infected | SAFV-1 | 3 | 0 | 5 | 3 | 2 | 8 |
| SAFV-2 | 9 | 4 | 28 | 13 | 15 | 41 |
| SAFV-3 | 1 | 5 | 23 | 12 | 11 | 29 |
| SAFV-6 | 0 | 0 | 4 | 1 | 3 | 4 |
| Untyped | 4 | 3 | 26 | 11 | 15 | 33 |
| Single-detection | 3 (17.6) | 3 (25.0) | 28 (32.6) | 10 (25.0) | 18 (39.1) | 34 (29.6) | 0.459 |
| Co-detection | 14 (82.4) | 9 (75.0) | 58 (67.4) | 30 (75.0) | 28 (60.9) | 81 (70.4) |

**NOTE:** HFMD, Hand, foot, and mouth disease. The P value was calculated by comparing cohort 1, cohort 2 and cohort 3 as a general.

**SAFV strains accession numbers.** The sequences obtained from the study were submitted to NCBI with the GenBank Accession Numbers KJ944637–KJ944718. The genomic sequences were assembled using Lasergene’s DNA SeqMan software (version 7.1.0, DNA Star Inc. Madison, WI, USA). The MEGA program (version 6.0, Sudhir Kumar, Arizona State University) was used for alignments and phylogenetic tree construction by maximum likelihood method using 1000 bootstrap pseudo replicates. Nucleotide identities among strains were calculated using BioEdit (version 7.1.3, www.mbio.ncsu.edu/bioedit/bioedit.html).

**Statistical analysis.** Descriptive statistics were performed, with continuous variables summarized as median and range, and categorical variables summarized as frequencies and proportions. The statistical significance between various groups was tested using the χ² test or Fisher exact test for categorical variables and independent t test or nonparametric test for continuous variables. A two-sided P value of less than 0.05 was considered to be statistically significant. Analyses were performed using SPSS version 11.5 (SPSS).

**Results**

Detection and genotyping of SAFV in three cohorts of patients. A total of 6052 patients (1647, 2013, and 2392 patients in cohorts 1, 2, and 3, respectively) were recruited into the study, 3810 (62.9%) of which were boys. Ages ranged from 26 days to 14 years (median, 12 months). More boys were included in cohort 1 (P = 0.044), and older age was presented in cohort 3 (P < 0.001) (Table 1).

Among all the tested patients, 115 (1.9%) were SAFV-positive, 17 (1.0%), 12 (0.6%), and 86 (3.6%) from cohorts 1, 2, and 3, respectively (Table 1). A significantly higher detection rate was found in HFMD patients among the three cohorts (P < 0.001). A total of 911 specimens (352 throat swab, 423 sera, and 136 stools) were collected from asymptomatic children. The age ranged from 4 months to 78 months (median, 29 months), and 553 (60.7%) were boys. No positive detection was found in any type of samples from asymptomatic children.

From the 115 SAFV-positive patients, 82 patients were successfully sequenced for VP1 nucleotide sequences, and four genotypes were identified: SAFV-1 (8, 9.8%), SAFV-2 (41, 50.0%), SAFV-3 (29,
Demographic and clinical characteristics of the SAFV-positive patients. The median age of the SAFV-positive patients was 24 months, and ranged from two months to 108 months. Of these patients, 70.4% were males. In cohort 1, the SAFV-positive patients were significantly older than the SAFV-negative patients (36.0 months vs. 9.0 months, \( P < 0.001 \); Table 2). The infection rate increased significantly with age among children below 6 years old (Cochran-Armitage trend test \( = -5.21, P < 0.001 \)). The highest positive rate (4.3%) was found in 4- to 5-year-old patients (Supplemental Table 1). In cohorts 2 and 3, the age and gender profiles were highly comparable between the SAFV-positive and SAFV-negative patients (all \( P > 0.05 \)).

In cohort 1, seven (41.2%) of the SAFV-positive patients, which is significantly higher than that of the SAFV-negative patients (41.2% vs. 14.2%, \( P = 0.002 \)), developed asthma that were related with this disease episode. By contrast, the opposite trend was found for pneumonia (47.1% vs. 73.4%, \( P = 0.015 \)). Other clinical manifestations were highly comparable between SAFV-positive and SAFV-negative patients (all \( P > 0.05 \)). In cohort 2, the diarrhea duration was comparable between SAFV-positive and SAFV-negative patients. No SAFV-positive patients exhibited symptoms of vomiting, but 928 (46.3%) SAFV-negative patients did (\( P = 0.009 \)). Within cohort 3, the severe HFMD patients had significantly higher SAFV-positive rate than the mild HFMD patients (5.2% vs. 2.6%, \( P = 0.001 \)). Furthermore, SAFV-positive patients presented with significantly higher frequency of nervous system manifestation than the SAFV-negative patients (52.3% vs. 37.1%, \( P = 0.004 \)).

### Table 2 | The demographic and clinical characteristics of the SAFV infected patients

| Variable                      | SAFV-positive | SAFV-negative | P value |
|-------------------------------|---------------|---------------|---------|
|                               | Subtotal      | Single-detection | Co-detection | SAFV-positive vs. SAFV-negative | Single-detection vs. co-detection |
| Cohort 1                      | (n = 17)      | (n = 3)        | (n = 14)   | (n = 1630)                      |                                   |
| Age, months (median, range)   | 36 (8–108)    | 45 (38–108)    | 31 (8–77)  | 9 (1–172)                       | \( <0.001^a \)                     |
| Sex, boy (%)                  | 12 (70.6)     | 2 (66.7)       | 10 (71.4)  | 1084 (66.5)                     | 0.723<sup>a</sup>                  |
| Clinical manifestation        |               |               |            |                                  | 1.00<sup>a</sup>                  |
| Cough                         | 16 (94.1)     | 3 (100)        | 13 (92.9)  | 1534 (94.1)                     | 1.00<sup>b</sup>                  |
| Pneumonia                     | 8 (47.1)      | 1 (100)        | 5 (38.5)   | 1196 (73.4)                     | 0.015<sup>b</sup>                 |
| Asthma                        | 9 (52.9)      | 0 (0)          | 9 (64.3)   | 231 (14.2)                      | 0.015<sup>b</sup>                 |
| Rhinorrhea                    | 7 (41.2)      | 0 (0)          | 7 (50.0)   | 231 (14.2)                      | 0.015<sup>b</sup>                 |
| Bronchitis                    | 6 (35.3)      | 0 (0)          | 6 (42.9)   | 749 (46.0)                      | 0.380<sup>b</sup>                 |
| Diarrhea times \( \geq 5 \)   | 2 (13.0)      | 0 (0)          | 2 (13.0)   | 299 (18.3)                      | 0.752<sup>b</sup>                 |
| Vomiting                      | 0 (0)         | 0 (0)          | 0 (0)      | 928 (46.3)                      | 0.009<sup>b</sup>                 |
| Cohort 2                      | (n = 12)      | (n = 3)        | (n = 9)    | (n = 2001)                      |                                   |
| Age, months (median, range)   | 11 (2–38)     | 13 (11–14)     | 10 (2–37)  | 10 (1–1630)                     | 0.774<sup>a</sup>                 |
| Sex, boy (%)                  | 9 (75.0)      | 2 (66.7)       | 7 (77.8)   | 1246 (62.2)                     | 0.552<sup>b</sup>                 |
| Clinical manifestations       |               |               |            |                                  | 0.618<sup>b</sup>                 |
| Diarrhea times \( \geq 5 \)   | 2 (13.0)      | 1 (100)        | 1 (11.1)   | 974 (48.6)                      | 0.290<sup>b</sup>                 |
| Vomiting                      | 0 (0)         | 0 (0)          | 0 (0)      | 928 (46.3)                      | NA                                |
| Cohort 3                      | (n = 86)      | (n = 28)       | (n = 58)   | (n = 2306)                      |                                   |
| Age, months (median, range)   | 24 (6–55)     | 20 (9–55)      | 24 (6–52)  | 24 (1–168)                      | 0.335<sup>a</sup>                 |
| Sex, boy (%)                  | 60 (69.8)     | 20 (71.4)      | 40 (69.0)  | 1537 (66.7)                     | 0.547<sup>b</sup>                 |
| Outcome                       |               |               |            |                                  | 0.816<sup>b</sup>                 |
| Mild HFMD                     | 39 (45.4)     | 10 (35.7)      | 29 (50.0)  | 1450 (62.9)                     | \( <0.001^b \)                     |
| Severe HFMD                   | 47 (54.7)     | 18 (64.3)      | 29 (50.0)  | 856 (37.1)                      | 0.218<sup>b</sup>                 |
| Clinical manifestations       |               |               |            |                                  | 0.607<sup>b</sup>                 |
| Respiratory system            | 8 (9.3)       | 2 (7.1)        | 6 (20.3)   | 199 (8.6)                       | 0.828<sup>b</sup>                 |
| Digestive system              | 4 (4.7)       | 3 (10.7)       | 1 (1.7)    | 134 (5.8)                       | 0.816<sup>b</sup>                 |
| Circulatory system            | 4 (4.7)       | 1 (3.6)        | 3 (5.2)    | 193 (8.4)                       | 0.218<sup>b</sup>                 |
| Nervous system                | 45 (52.3)     | 17 (60.7)      | 28 (48.3)  | 856 (37.1)                      | 0.004<sup>b</sup>                 |
| Note: respiratory system syndromes were defined as the presence of at least one of the following: cough, bronchitis or other upper respiratory tract disease, or pneumonia; digestive system syndromes were defined as the presence of at least one of the following: diarrea or vomit; circulatory system syndromes were defined as the presence of at least one of the following: myocarditis or cardiac damage; nervous syndromes were defined as the presence of at least one of the following: meningitis, encephalitis, brain myelitis, coma, acute flaccid paralysis or seizures. HFMD, Hand, foot, and mouth disease; NA, not applicable.

<sup>a</sup>Fisher exact test.  
<sup>b</sup>Mann-Whitney U test.  
<sup>c</sup>chi-squared test, two-sided.
eight SAFV-positive sera were successfully identified to be SAFV-2 confirmed by nested RT-PCR targeting the 5’-UTR. Two out of six SAFV-positive CSFs were successfully sequenced for VP1. The strains from the present study were grouped into three sublineages. All the SAFV-1 cases from the current study were grouped into the Chongqing sublineage, regardless of the cohort group. In SAFV-2, five lineages were constructed, which included European, North American, Middle-Eastern, Japanese, and East Asian lineages (Figure 2B). The strains from the present study were grouped into East Asian lineage, which, however, diverged from those from Beijing or Thai strains. In SAFV-3, six lineages were formed depending on the geographical origin. The Chongqing strains in this study were classified into the Asian lineages, forming a sublineage that was distinct from the strains of other regions (Figure 2C). In SAFV-6, the strains from three locations were classified into three clusters. The Chongqing strains formed a separate cluster, which is different from those of Japanese and Pakistani strains (Figure 2D).

Detection of SAFV in sera and CSFs. Additional 171 CSFs were collected from patients with HFMD-associated encephalitis, and six (3.5%) were found to be SAFV-6 positive using real-time RT-PCR, and five were further confirmed by nested RT-PCR targeting the 5’-UTR. Two of six SAFV-6 positive CSFs were successfully sequenced for VP1, and only SAFV-3 was identified. Among 760 serum samples collected from HFMD patients (355 from mild HFMD patients and 328 from severe HFMD patients), eight HFMD patients (3.5%) were found to be SAFV-positive using real-time RT-PCR, and five were further confirmed by nested RT-PCR targeting the 5’-UTR. Two out of eight SAFV-positive sera were successfully identified to be SAFV-2 and all from patients with HFMD-associated encephalitis. From three of the six SAFV-positive CSFs and four of eight SAFV-positive sera, no HEV was detected.

### Discussion

Information on the potential disease associations of SAFV has increased over the past six years, with data concentrated particularly on the involvement of SAFV in pediatric respiratory and gastrointestinal tract infections. In the current study, the prevalence of SAFV in pediatric patients in the recent four years in Chongqing, China was determined by performing molecular epidemiological investigation on three cohorts of patients. In addition to ARTI and diarrhea patients, an unexpected significantly higher prevalence of SAFV was found in HFMD patients, especially in patients with HFMD-associated encephalitis. Multiple genotypes of SAFV, which displayed geographic specific distribution pattern, were also found to be co-circulating in China. These data provided a reliable estimation based on a large sample size and long observation period, thereby expanding the understanding on the epidemiological features of SAFV.

One might query the higher frequency of SAFV in the HFMD patients was due to the old age and more sampling types of this cohort, in comparison with the ARTI and diarrhea cohorts. An increasing prevalence of SAFV was found in ARTI patients, but not in the HFMD patients. Thus, the age mismatch among the three cohorts might play minor roles in determining the detection rates. On the other hand, although the HFMD cohort was tested using stool, respiratory, blood, and CSF samples, an over-estimation of infection rate was not generated, because stool samples offered the highest diagnostic sensitivity. When we took no account of the patients with stool negative while other sample types are positive, a prevalence rate of 3.1% was still obtained. Thus, this effect alone might not explain the higher prevalence observed in HFMD.

Several lines of evidence indicate that SAFV might act as the agent responsible for the clinical syndromes, at least for some of the subjects. First, although co-detection with other viruses existed, SAFVs were the only pathogens identified in 34 (29.6%) SAFV-positive specimens in this study. The co-detection of SAFV with other viruses ranged from 0% to 100% in previous studies, and the variation in results is probably due to different diagnostic assays applied. In the present study, 15 common respiratory viruses in ARTI patients, 9 common viruses in diarrhea patients, and all EVs in HFMD patients were detected. The current results might represent a higher probability of single infection than the previous studies. Still, single-infection by SAFV is only true relative to the list of viruses that are being tested. Second, the present study also revealed the negative detection of SAFV in healthy children, indicating that SAFV is less likely to be present in asymptomatic subjects. This result is consistent with a previous study that was conducted among 39 asymptomatic patients in northern Germany. Finally, more severe clinical outcome and manifestation of nervous system were exhibited by SAFV-positive HFMD patients than in SAFV-negative HFMD patients.

In our study, SAFVs were co-detected with other viruses in 14 (82.4%), 9 (75.0%), and 28 (32.6) specimens in ARTI, diarrhea and
HFMD patients respectively. For ARTI and diarrhea cohorts, no differential clinical manifestations were inferred from the patients with SAFV single detection or SAFV co-detection, probably due to small sample size for comparison. However, for HFMD patients, we found the co-detection of SAFV could aggravate the clinical outcome of EV71 infection. We are uncertain whether SAFV/EV71 co-detection can lead to synergies in the HFMD pathogenic mechanism, and therefore responsible for the pathogenesis of severe HFMD, which need further investigation in the future.

Previous studies have demonstrated the existence of SAFV in CSF, but it was only limited to the case report. In 2011, SAFV-3 was isolated from the CSF sample of a 9-year old boy with aseptic meningitis. SAFV-2 was subsequently detected in the CSF and fecal samples of one child with cerebellitis as well as in the CSF, blood, and myocardium of another child who died suddenly with no history of illness. In the current study, the existence of SAFV-3 was observed in the CSF and SAFV-2 was observed in serum samples from the patients with HFMD-associated encephalitis. Furthermore, among half of the SAFV-positive CSFs and sera, SAFV was the solely detected pathogen. This information provided the first valid estimation of SAFV in pediatric encephalitis.

Pediatric encephalitis caused by viral infection has always been of great public health concern worldwide because of its high morbidity and mortality. The common agents of viral encephalitis include herpes simplex virus 1, varicella zoster virus, EV, Epstein-Barr virus, human herpesvirus 6, and measles virus. However, an etiological diagnosis was reached in less than 50% of the cases despite the use of modern microbiological and radiological methods. The list of uncommonly observed etiological agents for pediatric encephalitis remained far from complete. Animal experiments have suggested...
that SAFV-2 and SAFV-3 can cause serious invasive infections and are neurotropic in mice. Hertzler et al. reported that high doses of SAFV-2 intraperitoneally inoculated into adult mice resulted in paralysis and neuropathological changes consistent with acute encephalomyelitis, particularly in the limbic system. Sorgeloss et al. showed that both SAFV-2 and SAFV-3 can infect the heart and the central nervous system of 129/Sv mice. Furthermore, they found that SAFV-3 was more neurotropic than SAFV-2, and intracerebral inoculation of SAFV-3 into FVB/n mice caused acute encephalitis. In the current study, the existence of SAFV-2 and SAFV-3 was observed in the patients with HFMD-associated encephalitis. The present results, together with those of the earlier studies, indicate that SAFV-2 and SAFV-3 might play a role in the pathogenesis of viral encephalitis. However, these results warrant further confirmation by independent studies.

To the best of the authors’ knowledge, this study is the first to document SAFV in HFMD patients. The high frequency of SAFV in the stools, CSFs, and sera of children with HFMD and the absence of SAFV in healthy children suggests that this new virus might contribute to HFMD, especially among severe HFMD patients. Although with various patient types and large sample size, the major findings in the present study, especially the causal relationship between SAFV and the clinical disease, should be confirmed by serological study in the future.

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Acknowledgments

The authors would like thank all the subjects, their families, and collaborating clinicians for their participation. This work was supported by grants from the China Mega-Project on Infectious Disease Prevention (No. 2013ZX10004202), the National Natural Science Foundation (No. 81222037) and the Military Medical and Technology Twelfth Five-Year Science and Research Key Plan (BWS11C073).

Author contributions

W.L., W.C.C., X.A.Z. and Q.B.L. designed the study. H.M.X. and E.M.L. were responsible for recruitment of subjects. X.A.Z., Q.B.L., Y.W., J.Z., D.D.H. and C.T.G. performed experiments. W.L., W.C.C., X.A.Z. and Q.B.L. conducted data management, performed statistical analyses, interpreted results and wrote the manuscript. All authors reviewed the manuscript.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/srep. Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Zhang, X.-A. et al. Prevalence and genetic characteristics of Saffold cardiovirus in China from 2009 to 2012. *Sci. Rep.* **5**, 7704; DOI:10.1038/srep07704 (2015).