

Possible pandemic threat from new reassortment of influenza A(H7N9) virus in China

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Citation style for this article:

Meng Z, Han R, Hu Y, Yuan Z, Jiang S, Zhang X, Xu J. Possible pandemic threat from new reassortment of influenza A(H7N9) virus in China. *Euro Surveill.* 2014;19(6):pii=20699. Available online: <http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20699>

Article submitted on 29 January 2014 / published on 13 February 2014

Avian influenza A(H7N9) virus re-emerged in China in December 2013, after a decrease in the number of new cases during the preceding six months. Reassortment between influenza A(H7N9) and local H9N2 strains has spread from China's south-east coast to other regions. Three new reassortments of A(H7N9) virus were identified by phylogenetic analysis: between A(H7N9) and Zhejiang-derived strains, Guangdong/Hong Kong-derived strains or Hunan-derived A(H9N2) strains. Our findings suggest there is a possible risk that a pandemic could develop.

Recent re-emerged influenza A(H7N9) virus infections in China – especially the rapid outbreak in Zhejiang province in December 2013, involving 60 cases [1] – have raised concerns. Although several reports described the genetic characteristics of the virus [2-4], little is known about its further evolution after the initial outbreak in March 2013 [2] and the current re-emergence. As of 31 January 2014, there were a total of 260 cases: 127 of these have occurred in 2014 [5,6]. Cases have been reported from Zhejiang, Guangdong and Jiangsu provinces, Shanghai metropolitan area and Hong Kong in 2014 [6].

It is important to know whether new variants or lineages of influenza A(H7N9) virus are responsible for this re-emergence of the virus. In this study, four lineages and three new reassortments of A(H7N9) virus were identified by phylogenetic analysis and DNA mutation analysis of the PB1 gene.

Sequences analysis of PB1 genes from influenza A(H7N9) virus isolates

We retrieved 72 PB1 gene sequences of influenza A(H7N9) viruses, isolated from 11 Chinese provinces and cities, from the EpiFlu database of the Global Initiative on Sharing Avian Influenza Data (GISAID) deposited from March 2013 to January 2014 (Tables 1 and 2). In particular, the most recent A(H7N9) virus isolates from Hong Kong were also retrieved, through GISAID (A/Hong Kong/5942/2013 in November 2013

and A/Hong Kong/734/2014 in January 2014). We carried out a Basic Local Alignment Search Tool (BLAST) search to acquire related reference sequences in the National Center for Biotechnology Information (NCBI) Influenza Virus Resource [7]. Multiple alignments of sequences of eight genes of A(H7N9) virus isolates (PB2, PB1, PA, HA, NP, NA, MP, NS) were made using Bio-Edit7.0 software. We then carried out a phylogenetic analysis using MEGA6.1, as previously described [8,9].

In order to generate a neighbor-joining tree, the statistical robustness of the tree and the reliability of the branching patterns were confirmed by bootstrapping (1,000 replicates) and the effective transmission linkage was supported by a bootstrap value over 80% at the tree node. In accordance with previous studies reporting the virus as a triple reassortant A(H7N9) [2-4], we also observed that all A(H7N9) virus strains analysed, including the latest strains from Hong Kong (Hong Kong strains 5942 and 734), were part of one large cluster in an HA and NA gene-derived neighbor-joining tree (data not shown). However, analysis of six internal genes originating from influenza A(H9N2) virus identified multiple effective A(H7N9) clusters in PB2, PB1, NP, MP gene-derived neighbor-joining trees. As previously described, there is frequent PB2-PB1-PA-NP co-segregation during avian influenza virus reassortment [10]. Clusters of A(H7N9) consistent with this were observed in PB2, PB1 and NP gene-derived neighbor-joining trees (data not shown). Therefore, we then performed further phylogenetic analysis of A(H7N9) and A(H9N2) PB1 gene sequences.

At least four distinct clusters of A(H7N9) virus isolates were identified in a PB1 gene-derived neighbor-joining tree by high bootstrap value (>80%) (Figure 1). Cluster 1 containing poultry- or human-derived A(H7N9) virus isolates represents the earliest infections (shown by the collection date in Tables 1 and 2) and covers the majority of A(H7N9) virus infections in 2013, while the

TABLE 1A

Information on influenza A(H7N9) viruses in four distinct clusters, China, March 2013–January 2014

Cluster	Isolate name	Location	Host	Collection date
1	A/Shanghai/3/2013	Shanghai		2013-Feb-27
	A/Shanghai/4664T/2013	Shanghai		2013-Mar-5
	A/Anhui/1/2013	Anhui		2013-Mar-20
	A/Changsha/1/2013	Hunan		2013-Mar-22
	A/Hangzhou/1/2013	Zhejiang		2013-Mar-24
	A/Hangzhou/2/2013	Zhejiang		2013-Mar-25
	A/chicken/Anhui-Chuzhou/01/2013	Anhui		2013-Mar-29
	A/environment/Nanjing/2913/2013	Jiangsu	Envir	2013-Mar-29
	A/Jiangsu/01/2013	Jiangsu		2013-Mar-30
	A/Wuxi/1/2013	Jiangsu		2013-Mar-31
	A/Wuxi/2/2013	Jiangsu		2013-Mar-31
	A/chicken/Shanghai/017/2013	Shanghai	Chicken	2013-Apr
	A/chicken/Zhejiang/DTID-ZJU01/2013	Zhejiang	Chicken	2013-Apr
	A/environment/Wuxi/1/2013	Jiangsu	Envir	2013-Apr-2
	A/Environment/Shanghai/S1088/2013	Shanghai	Envir	2013-Apr-3
	A/Environment/Shanghai/S1438/2013	Shanghai	Envir	2013-Apr-3
	A/Environment/Shanghai/S1439/2013	Shanghai	Envir	2013-Apr-3
	A/pigeon/Shanghai/S1421/2013	Shanghai	Pigeon	2013-Apr-3
	A/pigeon/Shanghai/S1423/2013	Shanghai	Pigeon	2013-Apr-3
	A/Zhejiang/02/2013	Zhejiang		2013-Apr-3
	A/Zhejiang/DTID-ZJU01/2013	Zhejiang		2013-Apr-3
	A/environment/Hangzhou/34-1/2013	Zhejiang	Envir	2013-Apr-4
	A/Jiangsu/04/2013	Jiangsu		2013-Apr-5
	A/Shanghai/9/2013	Shanghai		2013-Apr-8
	A/Jiangsu/09/2013	Jiangsu		2013-Apr-9
	A/Shanghai/10/2013	Shanghai		2013-Apr-9
	A/Jiangsu/06/2013	Jiangsu		2013-Apr-10
	A/chicken/Zhejiang/SD033/2013	Zhejiang	Chicken	2013-Apr-11
	A/Beijing/01-A/2013	Beijing		2013-Apr-12
	A/Anhui/02/2013	Anhui		2013-Apr-14
	A/chicken/Jiangsu/S002/2013	Jiangsu	Chicken	2013-Apr-16
	A/chicken/Jiangsu/SC035/2013	Jiangsu	Chicken	2013-Apr-16
	A/chicken/Jiangsu/SC537/2013	Jiangsu	Chicken	2013-Apr-16
	A/Duck/Anhui/SC702/2013	Anhui	Duck	2013-Apr-16
	A/wildpigeon/Jiangsu/SD001/2013	Jiangsu	Pigeon	2013-Apr-17
	A/homingpigeon/Jiangsu/SD184/2013	Jiangsu	Pigeon	2013-Apr-20
	A/Anhui/03/2013	Anhui		2013-Apr-21
	A/Taiwan/S02076/2013	Taiwan		2013-Apr-22
	A/Taiwan/To2081/2013	Taiwan		2013-Apr-22
	A/Fujian/01/2013	Fujian		2013-Apr-23
	A/Fujian/1/2013	Fujian		2013-Apr-24
	A/Taiwan/1/2013	Taiwan		2013-Apr-24
	A/Environment/Guangdong/C13281025	Guangdong	Envir	2013-Apr-26
	A/Environment/Guangdong/C13281030	Guangdong	Envir	2013-Apr-26
	A/environment/Fujian/SC337/2013	Fujian	Envir	2013-Apr-30
	A/Zhejiang/DTID-ZJU10/2013	Zhejiang		2013-Oct-14
	A/shanghai/05/2013	Shanghai		2013-Apr-2

Envir: environment.

TABLE 1B

Information on influenza A(H7N9) viruses in four distinct clusters, China, March 2013–January 2014

Cluster	Isolate name	Location	Host	Collection date
2	A/chicken/Zhejiang/SD007/2013	Zhejiang	Chicken	2013-Apr-22
	A/environment/Hangzhou/37/2013	Zhejiang	Envir	2013-Apr-4
	A/chicken/Hangzhou/48-1/2013	Zhejiang	Chicken	2013-Apr-10
	A/environment/Hangzhou/109-1/2013	Zhejiang	Envir	2013-Apr-12
	A/Hangzhou/3/2013	Zhejiang		2013-Apr-2
3	A/Guangdong/1/2013	Guangdong		2013-Aug-10
	A/Duck/Zhejiang/SC410/2013	Zhejiang	Duck	2013-Apr-16
	A/chicken/Shanghai/S1080/2013	Shanghai	Chicken	2013-Apr-3
	A/HongKong/5942/2013	Hong Kong		2013-Nov-30
	A/HongKong/734/2014	Hong Kong		2014-Jan-7
4	A/chicken/Shanghai/019/2013	Shanghai	Chicken	2013-Apr-4
	A/Pigeon/Shanghai/S1069/2013	Shanghai	Pigeon	2013-Apr-2
	A/chicken/Shanghai/S1076/2013	Shanghai	Chicken	2013-Apr-3
	A/Shanghai/13/2013	Shanghai		2013-Apr-10
	A/environment/Henan/SC232/2013	Henan	Envir	2013-Apr-24
	A/Environment/Henan/SD429/2013	Henan	Envir	2013-Apr-24
	A/Jiangxi/01/2013	Jiangxi		2013-Apr-24
	A/Nanchang/1/2013	Jiangxi		2013-Apr-24
	A/Hunan/02/2013	Hunan		2013-Apr-25
	A/Environment/Shandong/1/2013	Shandong	Envir	2013-Apr-27
	A/chicken/Jiangxi/SD001/2013	Jiangxi	Chicken	2013-May-3
	A/Environment/Shandong/SD038/2013	Shandong	Envir	2013-May-3
	A/Shandong/01/2013	Shandong		2013-Apr-21
	A/Environment/Shandong/SD049/2013	Shandong	Envir	2013-May-3
	A/Hunan/01/2013	Hunan		2013-Apr-24

Envir: environment.

other three clusters indicate close phylogenetic links between A(H7N9) and A(H9N2) strains.

Generally, identification of distinct transmission clusters should meet the following criteria: a phylogenetic clade supported by both high bootstrap values (>80%) in a neighbor-joining tree and a posterior probability value of 1 at the Bayesian tree node [11,12]. For this purpose, a Bayesian phylogenetic inference was subsequently performed to confirm the distinct clusters of A(H7N9) isolates using MrBayes 3.1 as previously described [8,9]. As expected, the same four clusters (100% probability; posterior probability=1) were also seen in the Bayesian tree (Figure 2), as well as in the neighbor-joining tree (Figure 1), which further identified the effective transmission linkages inside these clusters (Figure 2).

Characterisation of transmission clusters

To further characterise the four transmission clusters of influenza A(H7N9) virus isolates, the mutation sites of the viral PB1 gene sequences were highlighted using

Nucleotide Sequences v2.2.3 (Figure3), revealing a distinct DNA mutation pattern of the four transmission clusters. Cluster1 shared the most common mutation sites with Shanghai-derived A(H7N9) strains, while all A(H7N9) strains from the other three clusters carried the most common mutation sites of their local A(H9N2) strains. The A(H7N9) strains of Cluster 2 carried the most common mutation sites of a Zhejiang-derived A(H9N2) strain, whereas the A(H7N9) strains in Clusters 3 and 4 had the most common mutation sites of Guangdong/Hong Kong-derived A(H9N2) and Hunan-derived A(H9N2) strains, respectively. These distinct DNA mutation patterns further identified new reassortments between A(H7N9) isolates and local A(H9N2) strains.

Phylogeographical trees of the influenza A virus PB1 gene sequences were constructed to further confirm the phylogenetic linkage of A(H7N9) and A(H9N2) virus strains using the BEAST V1.6.2 package as described previously [13,14]. The most recent common ancestor of the four clusters was estimated to be from

TABLE 2A

Origin of the influenza A(H7N9) viruses used for the analyses

Segment ID	Segment	Country	Collection date	Isolate name	Originating laboratory	Submitting laboratory	Authors
EPI447643	PB1	China	2013-Apr-14	A/Anhui/02/2013		WHO Chinese National Influenza Center	Wang D et al.
EPI447636	PB1	China	2013-Apr-21	A/Anhui/03/2013		WHO Chinese National Influenza Center	Wang D et al.
EPI439508	PB1	China	2013-Mar-20	A/Anhui/1/2013		WHO Chinese National Influenza Center	
EPI447836	PB1	China	2013-Apr-12	A/Beijing/01-A/2013		WHO Chinese National Influenza Center	Wang D et al.
EPI477319	PB1	China	2013-Mar-22	A/Changsha/1/2013		Other Database Import	Zhang RS et al.
EPI447889	PB1	China	2013-Mar-29	A/chicken/Anhui-Chuzhou/01/2013		WHO Chinese National Influenza Center	Wang D et al.
EPI443657	PB1	China	2013-Apr-10	A/chicken/Hangzhou/48-1/2013(H7N9)	Hangzhou Center for Disease Control and Prevention	Hangzhou Center for Disease Control and Prevention	Li J et al.
EPI457875	PB1	China	2013-Apr-16	A/chicken/Jiangsu/S002/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q et al.
EPI457867	PB1	China	2013-Apr-16	A/chicken/Jiangsu/SC035/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q et al.
EPI457851	PB1	China	2013-Apr-16	A/chicken/Jiangsu/SC537/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q et al.
EPI457843	PB1	China	2013-May-03	A/chicken/Jiangxi/SD001/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q et al.
EPI471846	PB1	China	2013-Apr-01	A/chicken/Shanghai/017/2013		Other Database Import	Yang D-Q et al.
EPI471847	PB1	China	2013-Apr-01	A/chicken/Shanghai/019/2013		Other Database Import	Yang D-Q et al.
EPI457827	PB1	China	2013-Apr-03	A/chicken/Shanghai/S1076/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q et al.
EPI457795	PB1	China	2013-Apr-03	A/chicken/Shanghai/S1080/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q et al.
EPI442719	PB1	China	2013-Apr-01	A/chicken/Zhejiang/DTID-ZJU01/2013		Other Database Import	Wu H et al.
EPI457763	PB1	China	2013-Apr-22	A/chicken/Zhejiang/SD007/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q et al.
EPI457747	PB1	China	2013-Apr-11	A/chicken/Zhejiang/SD033/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q et al.
EPI457739	PB1	China	2013-Apr-16	A/duck/Anhui/SC702/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q et al.
EPI457731	PB1	China	2013-Apr-16	A/duck/Zhejiang/SC410/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q et al.
EPI457723	PB1	China	2013-Apr-30	A/environment/Fujian/SC337/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q et al.
EPI490969	PB1	China	2013-Apr-26	A/Environment/Guangdong/C13281025/2013	Guangdong Provincial Center for Disease Control and Prevention	Guandong Centers for Disease Control	
EPI490977	PB1	China	2013-Apr-26	A/Environment/Guangdong/C13281030/2013	Guangdong Provincial Center for Disease Control and Prevention	Guandong Centers for Disease Control	
EPI443673	PB1	China	2013-Apr-12	A/environment/Hangzhou/109-1/2013(H7N9)	Hangzhou Center for Disease Control and Prevention	Hangzhou Center for Disease Control and Prevention	Li J et al.
EPI443570	PB1	China	2013-Apr-04	A/environment/Hangzhou/34-1/2013(H7N9)	Hangzhou Center for Disease Control and Prevention	Hangzhou Center for Disease Control and Prevention	Jing-Cao P

TABLE 2B

Origin of the influenza A(H7N9) viruses used for the analyses

Segment ID	Segment	Country	Collection date	Isolate name	Originating laboratory	Submitting laboratory	Authors
EPI443649	PB1	China	2013-Apr-04	A/environment/Hangzhou/37/2013	Hangzhou Center for Disease Control and Prevention	Hangzhou Center for Disease Control and Prevention	Li J et al.
EPI457715	PB1	China	2013-Apr-24	A/environment/Henan/SC232/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q et al.
EPI457707	PB1	China	2013-Apr-24	A/environment/Henan/SD429/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q et al.
EPI453621	PB1	China	2013-Mar-29	A/environment/Nanjing/2913/2013		Other Database Import	Bao C et al.
EPI447650	PB1	China	2013-Apr-27	A/Environment/Shandong/1/2013		WHO Chinese National Influenza Center	Wang D et al.
EPI457699	PB1	China	2013-May-03	A/environment/Shandong/SD038/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q et al.
EPI457683	PB1	China	2013-May-03	A/environment/Shandong/SD049/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q et al.
EPI440691	PB1	China	2013-Apr-03	A/Environment/Shanghai/S1088/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	
EPI457659	PB1	China	2013-Apr-03	A/environment/Shanghai/S1438/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q et al.
EPI457651	PB1	China	2013-Apr-03	A/environment/Shanghai/S1439/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q et al.
EPI467334	PB1	China	2013-Apr-02	A/environment/Wuxi/1/2013		Other Database Import	Qi X et al.
EPI447713	PB1	China	2013-Apr-23	A/Fujian/01/2013		WHO Chinese National Influenza Center	Wang D et al.
EPI453828	PB1	China	2013-Apr-24	A/Fujian/1/2013		Other Database Import	Weng Y et al.
EPI476699	PB1	China	2013-Aug-10	A/Guangdong/1/2013		Other Database Import	Guan W et al.
EPI441600	PB1	China	2013-Mar-24	A/Hangzhou/1/2013	Hangzhou Center for Disease Control and Prevention	Hangzhou Center for Disease Control and Prevention	Li J et al.
EPI446450	PB1	China	2013-Mar-25	A/Hangzhou/2/2013	Hangzhou Center for Disease Control and Prevention	Hangzhou Center for Disease Control and Prevention	Jing-Cao P
EPI446456	PB1	China	2013-Apr-02	A/Hangzhou/3/2013	Hangzhou Center for Disease Control and Prevention	Hangzhou Center for Disease Control and Prevention	Jing-Cao P
EPI457643	PB1	China	2013-Apr-20	A/homing pigeon/Jiangsu/SD184/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q et al.
EPI490880	PB1	Hong Kong (SAR)	2013-Nov-30	A/Hong Kong/5942/2013	Public Health Laboratory Services Branch, Centre for Health Protection	Public Health Laboratory Services Branch, Centre for Health Protection	Mak GC et al.
EPI498798	PB1	Hong Kong (SAR)	2014-Jan-07	A/Hong Kong/734/2014	Public Health Laboratory Services Branch, Centre for Health Protection	Public Health Laboratory Services Branch, Centre for Health Protection	Mak G et al.
EPI447699	PB1	China	2013-Apr-24	A/Hunan/01/2013		WHO Chinese National Influenza Center	Wang D et al.
EPI447692	PB1	China	2013-Apr-25	A/Hunan/02/2013		WHO Chinese National Influenza Center	Wang D et al.
EPI447920	PB1	China	2013-Mar-30	A/Jiangsu/01/2013		WHO Chinese National Influenza Center	Wang D et al.

TABLE 2C

Origin of the influenza A(H7N9) viruses used for the analyses

Segment ID	Segment	Country	Collection date	Isolate name	Originating laboratory	Submitting laboratory	Authors
EPI447850	PB1	China	2013-Apr-05	A/Jiangsu/04/2013		WHO Chinese National Influenza Center	Wang D et al.
EPI447678	PB1	China	2013-Apr-10	A/Jiangsu/06/2013		WHO Chinese National Influenza Center	Wang D et al.
EPI447657	PB1	China	2013-Apr-09	A/Jiangsu/09/2013		WHO Chinese National Influenza Center	Wang D et al.
EPI447706	PB1	China	2013-Apr-24	A/Jiangxi/01/2013		WHO Chinese National Influenza Center	Wang D et al.
EPI467775	PB1	China	2013-Apr-24	A/Nanchang/1/2013		Other Database Import	Zhou X et al.
EPI446699	PB1	China	2013-Apr-02	A/Pigeon/Shanghai/S1069/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	
EPI457635	PB1	China	2013-Apr-03	A/pigeon/Shanghai/S1421/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q et al.
EPI457627	PB1	China	2013-Apr-03	A/pigeon/Shanghai/S1423/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q et al.
EPI447720	PB1	China	2013-Apr-21	A/Shandong/01/2013		WHO Chinese National Influenza Center	Wang Dayan et al.
EPI447910	PB1	China	2013-Apr-02	A/shanghai/05/2013		WHO Chinese National Influenza Center	Wang Dayan et al.
EPI447809	PB1	China	2013-Apr-09	A/Shanghai/10/2013		WHO Chinese National Influenza Center	Wang Dayan et al.
EPI447784	PB1	China	2013-Apr-10	A/Shanghai/13/2013		WHO Chinese National Influenza Center	Wang Dayan et al.
EPI447960	PB1	China	2013-Feb-27	A/Shanghai/3/2013		WHO Chinese National Influenza Center	Wang Dayan et al.
EPI446964	PB1	China	2013-Mar-05	A/Shanghai/4664T/2013		Other Database Import	Hu Y
EPI447755	PB1	China	2013-Apr-08	A/Shanghai/9/2013		WHO Chinese National Influenza Center	Wang D et al.
EPI445910	PB1	Taiwan	2013-Apr-24	A/Taiwan/1/2013	National Influenza Center, Centers for Disease Control	Taiwan CDC	Ji-Rong Y et al.
EPI452258	PB1	Taiwan	2013-Apr-22	A/Taiwan/S02076/2013		Other Database Import	Chang SC et al.
EPI452266	PB1	Taiwan	2013-Apr-22	A/Taiwan/To2081/2013		Other Database Import	Chang SC et al.
EPI457619	PB1	China	2013-Apr-17	A/wild pigeon/Jiangsu/SD001/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q et al.
EPI467303	PB1	China	2013-Mar-31	A/Wuxi/1/2013		Other Database Import	Qi X et al.
EPI467311	PB1	China	2013-Mar-31	A/Wuxi/2/2013		Other Database Import	Qi X et al.
EPI447748	PB1	China	2013-Apr-03	A/Zhejiang/02/2013		WHO Chinese National Influenza Center	Wang D et al.
EPI441800	PB1	China	2013-Apr-03	A/Zhejiang/DTID-ZJU01/2013		Other Database Import	Chen H-L et al.
EPI477447	PB1	China	2013-Oct-14	A/Zhejiang/DTID-ZJU10/2013	The First Affiliated Hospital, College of Medicine, Zhejiang University	Shanghai Zhijiang Biotechnology Co., Ltd	Chen Y et al.

FIGURE 1

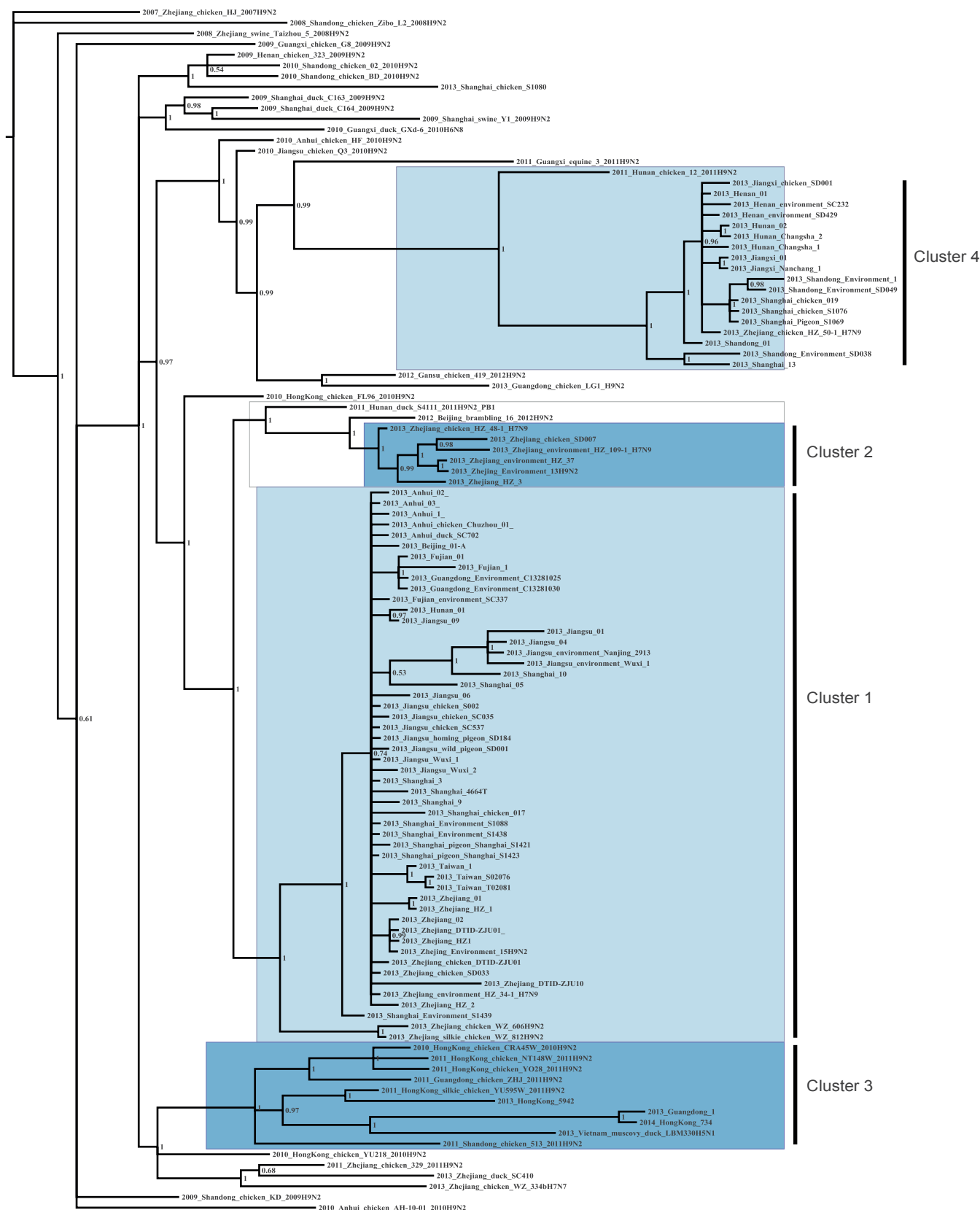
Neighbor-joining tree of PB1 gene sequences of influenza A(H7N9) and A(H9N2) viruses, China, March 2013–January 2014



The tree was constructed using MEGA6.1. Four distinct clusters supported by over 80% bootstrap probability were identified (subtrees with a thick black line). The A(H7N9) virus sequences of 2013 clustered with those of A(H9N2) strains with 100% bootstrap probability in three subtrees shown as Clusters 2–4. Notably, Guangdong- and Hong Kong-derived A(H7N9) sequences (empty triangles) showed a close transmission linkage with local A(H9N2) strains.

FIGURE 2

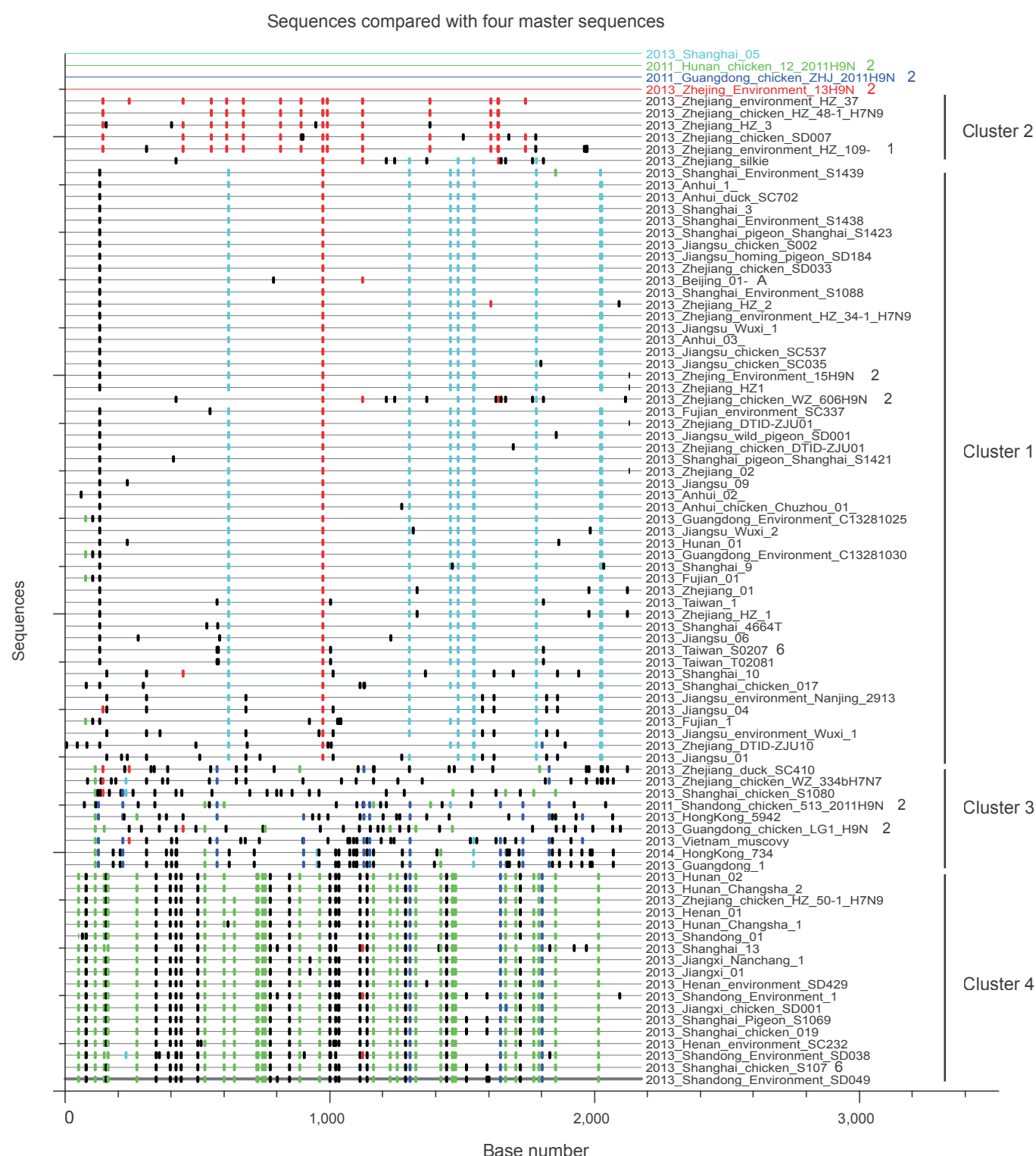
Bayesian tree of PB1 gene sequences of influenza A(H7N9) and A(H9N2) viruses, China, March 2013–January 2014



The tree was constructed using MrBayes 3.1. Significant linkages in Bayesian phylogenetic inference analysis were considered as those having posterior probabilities of 100%. Four transmission clusters (Clusters 1–4) with 100% posterior probability are indicated. Influenza A(H7N9) strains shows close transmission linkage with A(H9N2) strains in Clusters 2–4.

FIGURE 3

Highlighter analysis of mutation sites of influenzaA(H7N9) virus PB1 gene sequences, China, March 2013–January 2014



The analysis was carried out using Nucleotide Sequences v2.2.3. Four isolates (one from each cluster) were considered as master sequences based on the phylogenetic analysis: 2013_Shanghai_05 (cyan), 2011_Hunan_Chicken_12_H9N2 (green), 2011_Guangdong_Chicken_ZHJ_H9N2 (blue), 2013_Zhejiang_Environment_13H9N2 (red).

Maximum clade credibility trees of PB1 gene sequences of influenza A viruses, China, March 2013–January 2014

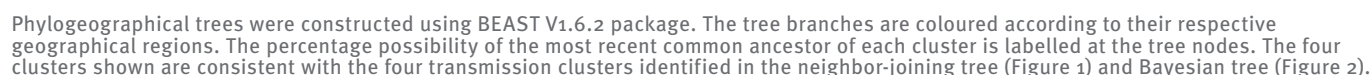
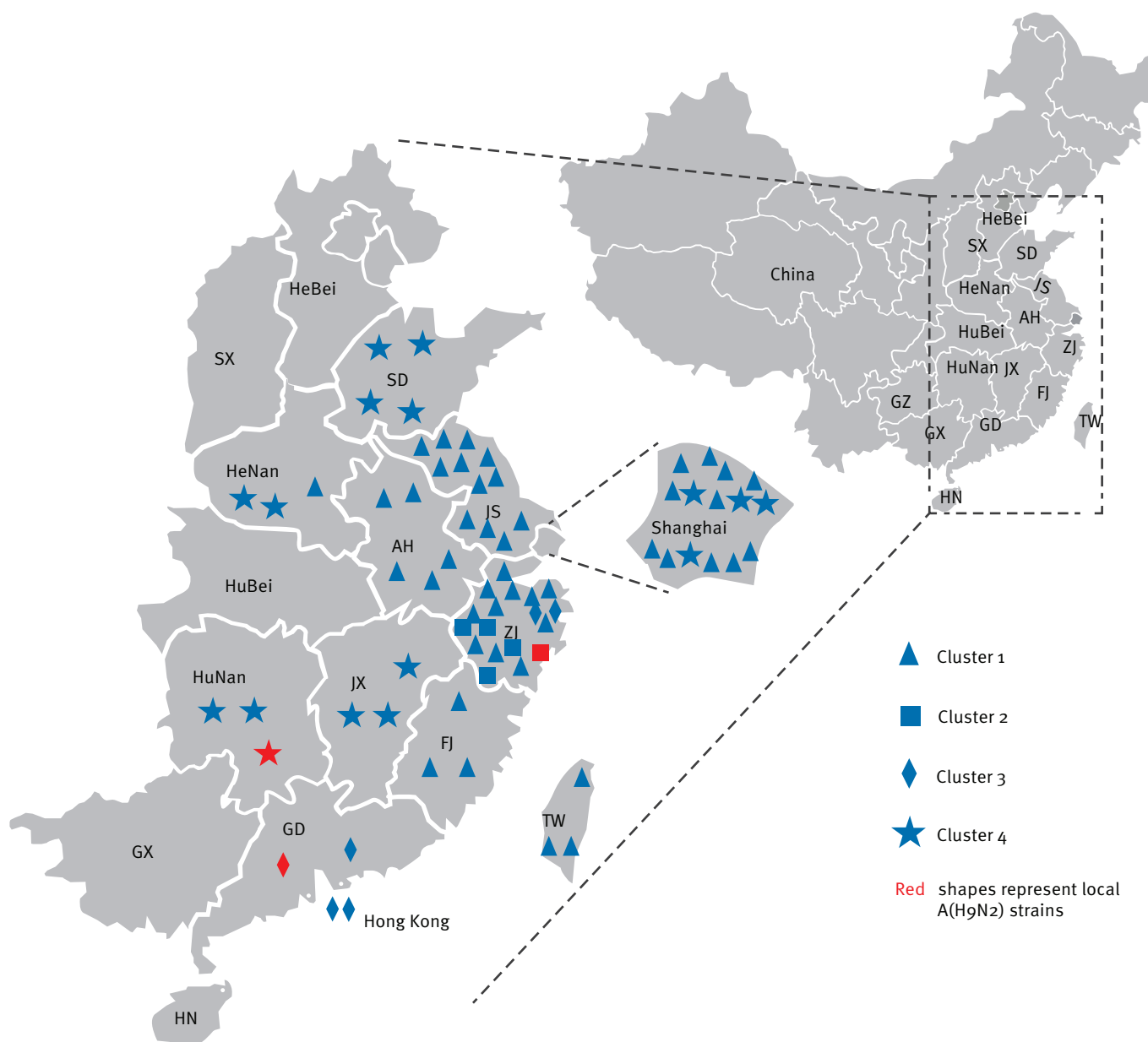


FIGURE 5

Geographical distribution of influenza A(H7N9) virus strains from four transmission clusters, China, March 2013–January 2014



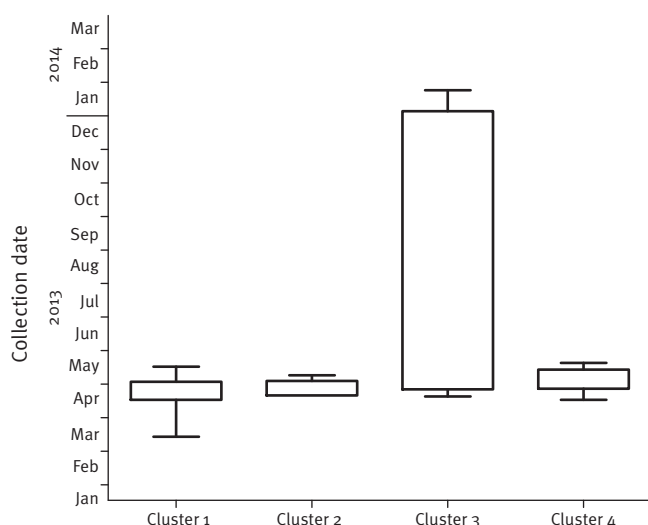
AH: Anhui; FJ: Fujian; GD: Guangdong; GX: Guangxi; HN: Hainan; JS: Jiangsu; JX: Jiangxi; SD: Shandong; SX: Shanxi; TW: Taiwan; ZJ: Zhejiang.

Shanghai-derived strains (Figure 4). In addition, the collection dates of Shanghai A(H7N9) strains were earlier than those of other strains in Clusters 1 and 4 (Tables 1 and 2), suggesting a critical role of Shanghai strains in dissemination of the virus (Figure 5). A new reassortment involving Zhejiang-derived A(H7N9) and Guangdong local A(H9N2) strains formed one independent cluster (Cluster 3), representing the latest and furthest A(H7N9) strains (from the earliest infecting strains in Shanghai) [2] (Figure 4). Moreover, as indicated by Cluster 4 in Figures 4 and 5, the new reassortment of Shanghai A(H7N9)- and Hunan A(H9N2)-derived strains may facilitate further dissemination of A(H7N9)

virus from the Yangtze River Delta Economic Zone (including Shanghai, Jiangsu, Zhejiang, Anhui) into neighbouring provinces (such as Shandong, Hunan, Henan and Jiangxi). Meanwhile, Shanghai and Zhejiang A(H7N9) strains were involved in Clusters 3 and 4, indicating that the new reassortment may occur in poultry. Moreover, both the time of the most recent common ancestor and collection date of strains in Clusters 2 and 4 (Figures 4 and 6) showed an obvious delay in comparison with those in Cluster 1, suggesting that reassortment probably occurred during the initial outbreaks. Unlike Clusters 3 and 4, Cluster 2 represents

FIGURE 6

Collection dates of influenza A(H7N9) virus isolates from four transmission clusters, China, March 2013–January 2014



The bars represent the standard deviation of the collection dates of the A(H7N9) strains in each cluster.

the reassortment between local A(H7N9) and A(H9N2) strains (both from Zhejiang).

Notably, the distinct time to the most recent common ancestor of Clusters 1, 2 and 4 (Figure 4) is consistent with the time course of collection date of the strains in the three clusters (Figure 6, Tables 1 and 2), suggesting distinct phases for transmission and reassortment of A(H7N9) virus in China. Cluster 1, with the earliest most recent common ancestor (Figure 4), may represent the first wave and main body of the A(H7N9) outbreak during first half of 2013, which facilitated the subsequent reassortment between A(H7N9) and local A(H9N2) strains, as Clusters 2 and 4 indicate. Additionally, although no time to the most recent common ancestor is indicated for Cluster 3 (Figure 4), all A(H7N9) strains in this cluster have been isolated very recently (Tables 1, and 2, Figure 6), which may represent the latest reassortment of A(H7N9) and A(H9N2) strains. The association between the expanding transmission and appearance of reassortments suggests a tendency for A(H7N9) evolution towards more and more geographical localisation. In addition, Shanghai or Zhejiang poultry-derived A(H7N9) strains may also play active roles in the process of reassortment and localisation (Tables 1 and 2).

Discussion

Our analysis revealed dynamic reassortments between influenza A(H7N9) and A(H9N2) viruses since the outbreak of A(H7N9) virus infection in March 2013. To some extent, the continuous transmission of H7N9 in Chinese

poultry has led to increasing diversity and new reassortment of A(H7N9) with local A(H9N2) strains. Our findings suggest that the re-emerged H7N9 infections may be triggered by new reassortment strains, such as those in the Guangdong/Hong Kong transmission of Cluster 3. In this regard, these infections may have implications for the traditional strategies of drug and vaccine development targeted against HA and NA genes [15]. In particular, the new reassortments generated by A(H7N9) and local A(H9N2) strains may produce avian influenza virus strains that are more adaptive and have a higher pathogenicity in humans [16], emphasising the importance of continuously monitoring the A(H7N9) epidemic.

To date, 127 cases of A(H7N9) virus infections have been reported in January 2014, almost the same number as reported in the spring of 2013 ($n=133$) [5,6]. Notably, Zhejiang and Guangdong provinces and the Shanghai metropolitan area, where new reassortment of A(H7N9) strains is being identified, have been the worst affected regions in China in 2014 [1,17,18]. Although the case-fatality rate in January 2014 (24%, 31/127) is not higher than that seen in the spring of 2013 (29%, 39/133) [5,6], the rapidly increasing number of cases of A(H7N9) virus infection in these three regions may raise concerns as to whether there is an association between circulation of the new A(H7N9) reassortment strains identified and accelerated transmission of A(H7N9) virus in humans. Therefore, it is of the utmost importance to monitor the risk of a potential pandemic initiated by various influenza virus strains.

Acknowledgments

We acknowledge the authors, originating and submitting laboratories of the sequences from GISAID's EpiFlu Database on which this research is based (see Table 2). All submitters of the data may be contacted directly via the GISAID website www.gisaid.org. This work was supported by Chinese National Grand Program on Key Infectious Disease Control (2012ZX10004-211), Shanghai Municipal Commission of Health and Family Planning (2013QLG003) and 985 program at Fudan University (EZF101606/018-019).

Conflict of interest

None declared.

Authors' contributions

Z.M. and J.X. conceived and designed the experiments. Z.M. performed the experiments and analysed the data. Z.Y., Y.H. and X.Z. contributed reagents/materials/analysis tools. Z.M. and R.H. wrote the paper.

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