Identification of a natural human serotype 3 parainfluenza virus

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Abstract
Parainfluenza virus is an important pathogen threatening the health of animals and human, which brings human many kinds of disease, especially lower respiratory tract infection involving infants and young children. In order to control the virus, it is necessary to fully understand the molecular basis resulting in the genetic diversity of the virus. Homologous recombination is one of mechanisms for the rapid change of genetic diversity. However, as a negative-strand virus, it is unknown whether the recombination can naturally take place in human PI. In this study, we isolated and identified a mosaic serotype 3 human PIV (HPV3) from in China, and also provided several putative PIV mosaics from previous reports to reveal that the recombination can naturally occur in the virus. In addition, two swine PIV3 isolates transferred from cattle to pigs were found to have mosaic genomes. These results suggest that homologous recombination can promote the genetic diversity and potentially bring some novel biologic characteristics of HPIV.

Introduction
Human parainfluenza virus (HPIV) is known to induce acute respiratory infections (ARI) including lower respiratory tract infection, which is a leading cause of morbidity and mortality in infants and young children [1,2]. Until now, four serotypes of parainfluenza virus (PIV) infecting human being have been found. Especially, human PIV (HPIV) 1, 2 and 3 are the second leading causative agents of pediatric hospitalizations due to respiratory disease following respiratory syncytial virus (RSV) [3]. It is important to know the mechanism resulting in genetic and antigenic diversity of HPIV for controlling the pathogen.

As one member of the Respirovirus genus of the family Paramyxoviridae, HPIV is an enveloped non-segmented single-stranded RNA virus [4]. RNA viruses usually exhibit genetic variation, which can be attributed to their high rate of mutation during their replication process and the large population size [5]. In addition, homologous recombination has been recognized increasingly as a potentially important means of generating and shaping genetic diversity in positive-strand RNA virus [6]. In several other members of the Paramyxoviridae family, Newcastle disease virus (NDV) [7-11] and human respiratory syncytial virus [12], natural recombinants have been detected. Moreover, attenuated vaccines were found to be able to influence the evolution process of NDV through exchanging their genetic material with circulating virus [7,9,11]. For HPIV, it is unknown whether there is natural recombinant virus circulating in the field.

In this study, we isolated and identified a natural type 3 HPIV mosaic isolate LZ22/FJ455842 (with a mosaic N gene) to show that homologous recombination can occur in HPIV3. Additionally, three HPIV1 isolates (HT88/U01082, HT89a/U01083 and HT89c/U01085) were found to be deposited in previous report. Interestingly, we also found that there were the two Swine PIV3 recombinants with mosaic L protein were thought to be associated with cross-species infection in previous report [13,14]. Collectively, these recombinant events suggested that homologous recombination played a role in HPIV genetic diversity and rapid evolution.

Results and Discussion
The sequence of LZ22 complete genome has been deposited in GenBank (Access Number, FJ455842). And the PIV3 complete genome sequence alignment dataset
was analyzed employing RDP3 software package for scanning the recombinant sequence. And the isolate LZ22 was found to have greatly strong recombination signal: RDP, p-value $< 10^{-21}$; GENECOVY, p-value $< 10^{-21}$; Bootscan, p-value $< 10^{-20}$, MaxChi p-value $< 10^{-3}$; Chimaera p-value $< 10^{-7}$; Siscan, p-value $< 10^{-8}$, 3Seq, p-value $< 10^{-13}$. And a breakpoint was located at position 485. Two strains GP and ZHYMgz01 were suggested as representatives of its putative parent lineages.

And then, Simplot software package was used to determine the recombination event [15]. Employing the Findsites subprogram of SimPlot, one potential breakpoint was located at parsimonious regions with the maximization of $\chi^2$, from positions 485 to 615 ($\chi^2 = 122.3$, $P < 0.0001$ of Fisher’s exact test). A similarity plot (Figure 1A) which was constructed by using all sites, revealed that the sequence of LZ22 showed greater affinity with one putative parent lineage of GP in the region from position 1 to 485 than the other putative parent ZHYMgz01 (100% versus 94%). However, sequence from positions 486 to 15536, ZHYMgz01 shared greater similarity with LZ22 than GP (98% versus 95%). $P$ value (Fisher’s Exact Test) and $\chi^2$ value of the breakpoint were shown on the vertical line in Figure 1. The identical evidence also appeared in BootScanning result (Figure 1B). The region from GP lineage spanned the amino terminal 1/3 of the N protein approximately.

At last, The phylogenic trees were also constructed using Mega 4 to determine the recombination events [16]. From positions 1 to 485, LZ22 and GP were clustered into the same sublineage with 98% bootstrap value, while ZHYMgz01 was grouped into distinct sublineage (Figure 2A). But, in the other portion, the arrangement of the phylogenetic tree reflecting the relationship of the three isolates was in contrast with the previous one (Figure 2B). The topology of the two phylogenetic trees around the breakpoint showed a significant statistic discrepancy when the mosaic was included.

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**Figure 1 (A, B) Results of Similarity and BootScanning analysis of the genome of LZ22_FJ455842.** The y-axis in Similarity plot (A) gives the percentage of sequence identity within a sliding window of 400 bp wide centered on the position plotted, with a step size between plots of 20 bp, while in BootScanning plot (B) represents the percentage of permuted trees. The $\chi^2$ of maximization and $P$ value of Fisher’s Exact test are shown near (or on) the vertical line. GP and ZHYMgz01 are used as two parental lineage sequences and JS_U51116 an outgroup sequence. The breakpoint is identified and located at position 485, with $\chi^2$ value maximized. The query sequence LZ22_FJ455842 demonstrates greater sequence identity and BootScanning support with GP in the beginning region while otherwise with ZHYMgz01_EU326526 in the complementary regions.
in analyzed data (Shimodaira-Hasegawa test, P < 0.001), constituting a powerful evidence for recombination.

The minor putative parent of LZ22 was isolated from Japan, suggesting the existence of a global reservoir of HPIV with local subreservoirs supporting extensive levels of virus circulation, which permitted co-infection and resulted in the recombination event at last. The potential breakpoint 485 presents in gene N coding nucleoprotein spanning the region from positions 56 to 1737 of genomic sequence [17]. In the previous study, viable artificial chimeric HPIV3 recombinants were constructed, which contained the nucleoprotein open reading frame (ORF) from either BPIV3 Kansas or shipping fever (SF) strain in place of the HPIV3 N ORF. The artificial recombinant (PIV3) in which the nucleocapsid N protein had been replaced by that of bovine PIV3 was found to be attenuated in primates [18]. Here, we isolated a HPIV3 with a natural mosaic in NP ORF. Interestingly, before the breakpoint, the similarity of peptide sequences was up to 98.5% (129/131) although the similarity of gene sequences was only 94% between the two putative parent lineages. It is unknown whether the recombination in N gene is also associated with their adaptation in host cell via a changed virulence.

In addition, since there has been no report to show homologous recombination can take place between PIVs before this study; we analyzed 55 isolates (Table 1) from GenBank in order to determine to what degree genetic diversity of the virus is affected by the homologous recombination. Five additional mosaic PIV sequences were detected through the analysis of sequences from isolates characterized in previous reports: HT88 (U01082) [19], HT89a [19] (U01083), HT89c (U01085) [19], 81-19252_Texas-81 (EU439429) [13,14] and 92-7783_ISU-92 (EU439428) [13,14] (Table 2). Two HN gene mosaics, HT88 and HT89a shared the same recombination event (Table 2), suggesting they descended from the same mosaic ancestor. Please also refer additional files 1, 2, 3, and 4 for the detail recombination information of each mosaic strain. These results suggested that homologous recombination did play a potential role in the evolution of the virus.

Interestingly, two mosaic viruses 81-19252_Texas-81 (U439429) and 92-7783_ISU-92 (EU439428) were reported to be involved in a cross infection between swine and bovine [13,14]. Both of the isolates had a mosaic L gene. The transcription and replication functions of the parainfluenza virus are associated with the large RNA polymerase protein. Additionally, polyadenylation, and RNA editing activities have to do with L protein [20]. The two putative mosaics were isolated from pigs in the United States [13,14] while both of their putative parent lineages (Shipping fever and 910N lineages) belonged to BPIV3. Viruses are largely species-specific with respect to their host and usually do not cross species boundaries [5]. Recombination processes will allow some viruses to acquire many of the key adaptive mutations in a single step, and thus make a major leap in fitness, which might result in a change of host tropism [21]. It might be necessary to further study.
whether the recombination event is relative to the BPIV3 cross-species infection.

In conclusion, this study provides the potential evidence that there is mosaic PIV in the field. Our observations show that homologous recombination is a molecular mechanism of PIV genetic diversity and evolution. Therefore, this study might be important for knowing the genetic basis resulting in the rapid change of PIV biologic characteristics.

Material and methods

Virus and sequencing

The virus LZ22 was isolated from lower respiratory tract of a patient infant with pneumonia in Lanzhou, Gansu Province of China in 2003. The virus was identified using previously described protocols [22]. After isolated, LZ22 was also purified 3 times by the plaque forming method in Vero cells. Before sequencing, LZ22 was passed 13 times and amplified in Vero cells for RT-PCR. Viral RNA was extracted from Vero cell virus cultures using RNAeasy mini kit (Qiagen, Netherlands) following the manufacturer's instructions. Reverse transcription was performed using SuperScript II one-step RT-PCR platinum Taq HiFi kit (Invitrogen, USA). 3' and 5' RACE were performed using 5'-full RACE CORE kit and 3'-RACE kit (Takala Dalian) to analyze the 3' and 5' UTR sequences. The primers of PCR and 3' and 5' RACE used in this study were listed in Table 3. The full genome of LZ22 was amplified and sequenced referring to previous report [22]. All PCR products were cloned into pGEM-T-vector (Promega USA) and sequenced by Takara Biotechnology (Dalian, China).

Recombination analysis

Compete genome and HN gene of PIV were retrieved from GenBank and aligned with CLUSTALW [23]. PIV3 compete genome and HPIV1 HN genes sequences analyzed in the study were listed in Table 1. Phylogenetic Neighbor-Joining (NJ) trees were set up by MEGA4 [16]. The nucleotide substitution models were optimized for Maximum-Likelihood (ML) trees employing jmodeltest (version 0.1.1) [24]. ML trees were constructed employing Phyml software with nucleotide substitution

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### Table 1 Typical PIV isolates used in the study

| GenBank Number | Strain   | Serotype | Host     | reference |
|----------------|----------|----------|----------|-----------|
| NC_001796      | unnamed  | HPIV3    | Human    | [29]      |
| A0812132       | GP       | HPIV3    | Guinea Pig | [30]  |
| EU439429       | 81-19252_{Texas-81} | SPV3 | Swine | [13]  |
| EU439428       | 92-7783_{ISU-92} | SPV3 | Swine | [13]  |
| FJ455842       | LZ22     | HPIV3    | Human    | This study |
| NC_002161      | unnamed  | BPIV3    | Bovine   | [31]      |
| EU424062       | 14702    | HPIV3    | Human    | Unpublished |
| EU326526       | ZHYMg01  | HPIV3    | Human    | Unpublished |
| D84095         | 910N     | BPIV3    | Bovine   | [32]      |
| AF178655       | Shipping Fever | BPIV3 | Bovine | [31]  |
| AF178654       | Kansas/152684 | BPIV3 | Bovine | [31]  |
| Z11575         | JS       | HPIV3    | Human    | [33]      |
| U51116         | JS       | HPIV3    | Human    | [34]      |
| EU277658       | Q5592    | BPIV3    | Bovine   | [35]      |
| U01082         | HT88     | HPIV1    | Human    | [19]      |
| U01083         | HT89a    | HPIV1    | Human    | [19]      |
| U01075         | HT82a    | HPIV1    | Human    | [19]      |
| U01074         | HT81b    | HPIV1    | Human    | [19]      |
| U01073         | HT81a    | HPIV1    | Human    | [19]      |
| U01076         | HT82b    | HPIV1    | Human    | [19]      |
| U01079         | HPIV1    | Human    | [19]      |
| U01078         | HPIV1    | Human    | [19]      |
| U01079         | HPIV1    | Human    | [19]      |
| U01084         | HPIV1    | Human    | [19]      |
| U01085         | HPIV1    | Human    | [19]      |
| U01086         | HPIV1    | Human    | [19]      |
| U01087         | HPIV1    | Human    | [19]      |
| M86785         | CH-B-73A | HPIV1    | Human    | [37]      |
| U070497        | Mil-69/91| HPIV1    | Human    | [36]      |
| M86786         | CH-B-73B | HPIV1    | Human    | [37]      |
| M86790         | CH-B-83A | HPIV1    | Human    | [37]      |
| U01079         | HT85a    | HPIV1    | Human    | [19]      |
| M86791         | CH-B-83B | HPIV1    | Human    | [37]      |
| M86789         | CH-B-79A | HPIV1    | Human    | [37]      |
| U01084         | HT86b    | HPIV1    | Human    | [19]      |
| U070498        | Mil-64/91| HPIV1    | Human    | [36]      |
| M86787         | CH-B-77  | HPIV1    | Human    | [37]      |
| U01081         | HT87     | HPIV1    | Human    | [19]      |
| M86784         | CH-B-70  | HPIV1    | Human    | [37]      |
| U01080         | HT85b    | HPIV1    | Human    | [19]      |
| M86784         | CH-B-70  | HPIV1    | Human    | [37]      |
| U01077         | HT83a    | HPIV1    | Human    | [19]      |
| U07044         | Mil-60/91| HPIV1    | Human    | [36]      |
| U01085         | HT89c    | HPIV1    | Human    | [19]      |
| U01078         | HT83b    | HPIV1    | Human    | [19]      |
| AF016280       | PIV1/    | HPIV1    | Human    | [38]      |
|                | Washington/20933/1964 | HPIV1 | Human |              |
| M86788         | CH-B-79A | HPIV1    | Human    | [37]      |
| U070939        | Mil-52/91| HPIV1    | Human    | [36]      |
| U070941        | Mil-54/91| HPIV1    | Human    | [36]      |
| M86781         | CH-A-80  | HPIV1    | Human    | [37]      |
| U070946        | Mil-62/91| HPIV1    | Human    | [36]      |
| U070942        | Mil-55/91| HPIV1    | Human    | [36]      |

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### Table 1 Typical PIV isolates used in the study (Continued)

| GenBank Number | Strain   | Serotype | Host     | reference |
|----------------|----------|----------|----------|-----------|
| U70945         | Mil-61/91| HPIV1    | Human    | [36]      |
| U70940         | Mil-53/91| HPIV1    | Human    | [36]      |
| M86783         | CH-A-81B | HPIV1    | Human    | [37]      |
| M86780         | CH-A-66  | HPIV1    | Human    | [37]      |
| M86782         | CH-A-81A | HPIV1    | Human    | [37]      |
| M31228         | unnamed  | HPIV1    | Human    | [39]      |
| M91648         | C39      | HPIV1    | Human    | [40]      |
| X55803         | unnamed  | HPIV1    | Human    | [40]      |
model of general time reversible model (GTR) and gamma distributed 4 (G4) [25], and displayed as graphics by using MEGA4 to determine the topology of each tree. Identification methods of homologous recombination were described as previous report [26,27]. Briefly, the sequence alignment files were sought for potential mosaic isolates using RDP software package [24,28]. The gene sequence similarity of mosaics and their putative parents were compared and displayed as graphics with Simplot software [15]. At last, incongruent phylogenetic relations of different gene regions delimited by crossover point were determined by phylogenetic trees.

### Table 2 Characteristics of PIV intragenic recombinants

| Strain     | $\chi^2_{\text{max}}$ | Simplot identified breakpoints | RDP identified breakpoints | Putative parent lineages | Z-score | P-value* |
|------------|------------------------|-------------------------------|---------------------------|--------------------------|---------|----------|
| LZ22       | 122.3                  | 485-615                       | 485                       | GP; Z1Hmgp01             | 9.24    | 2.3E-9   |
| 81-19252_Texas-81 | 366.9                | 8686-712                      | 8688                      | 910N; Shipping_Fever     | 9.34    | 2.5E-42  |
|            | 83.7                   | 12613-730                     | 12595                     | Shipping_Fever           | 6.45    | 3.5E-6   |
|            | 69.1                   | 13619-36                      | 13619                     | 14175                    |         |          |
|            | 62.6                   | 14134-245                     |                           |                          |         |          |
| 92-7783_ISU-92 | 179.7                  | 14134-206                     | 14137                     | 910N;                    | 9.52    | 1.3E-7   |
|            | 22.6                   | 14863-5071                    | 14989                     | Shipping_Fever           |         |          |
| HT88**     | 7.9; 8.5               | 300-396                       | 392; 839                  | 910N; Mil-49/91; HT89b   | 6.07    | 1.2E-7   |
| HT89a**    | 7.9; 8.5               | 300-396                       | 392; 839                  | 910N; Mil-49/91; HT89b   | 6.07    | 1.2E-7   |
| HT89c**    | 6.4; 14.2              | 351-392                       | 351; 767                  | Mil-58/91; Mil-51/91     | 5.98    | 3.8E-6   |

| Strain     | $\chi^2_{\text{max}}$ | Simplot identified breakpoints | RDP identified breakpoints | Putative parent lineages | Z-score | P-value* |
|------------|------------------------|-------------------------------|---------------------------|--------------------------|---------|----------|
| LZ22       | 122.3                  | 485-615                       | 485                       | GP; Z1Hmgp01             | 9.24    | 2.3E-9   |
| 81-19252_Texas-81 | 366.9                | 8686-712                      | 8688                      | 910N; Shipping_Fever     | 9.34    | 2.5E-42  |
|            | 83.7                   | 12613-730                     | 12595                     | Shipping_Fever           | 6.45    | 3.5E-6   |
|            | 69.1                   | 13619-36                      | 13619                     | 14175                    |         |          |
|            | 62.6                   | 14134-245                     |                           |                          |         |          |
| 92-7783_ISU-92 | 179.7                  | 14134-206                     | 14137                     | 910N;                    | 9.52    | 1.3E-7   |
|            | 22.6                   | 14863-5071                    | 14989                     | Shipping_Fever           |         |          |
| HT88**     | 7.9; 8.5               | 300-396                       | 392; 839                  | 910N; Mil-49/91; HT89b   | 6.07    | 1.2E-7   |
| HT89a**    | 7.9; 8.5               | 300-396                       | 392; 839                  | 910N; Mil-49/91; HT89b   | 6.07    | 1.2E-7   |
| HT89c**    | 6.4; 14.2              | 351-392                       | 351; 767                  | Mil-58/91; Mil-51/91     | 5.98    | 3.8E-6   |

Table 2 Characteristics of PIV intragenic recombinants

Table 3 Primers for sequencing of genome of HPIV-3

| Fragment (Positions) | Primer (5’-3’) | Sequence |
|----------------------|---------------|----------|
| NP NPS (1-1843)      | NPA           | TTCCTCTCCTCCAAGATCCATGATTIAGG |
| PP PPS (1616-3485)   | PPA           | CGTGTCAATGACCTGGTGATGTAATGG |
| M MS (3452-5183)     | MA            | GCCCGCGGATAGACGATCTGTCATGACAAAGACG |
| F FS (4724-7104)     | FA            | TGGCACCGTTGAACTCAAACGCTGCCCTTATG |
| HN HNS (6644-8744)   | HNA           | TGTGAATTTGTGCTATTCACCTTTTAAAGC |
| L1 L1S (8489-10684)  | L1A           | TGGTCAAAACAGAAGATCCAAAAAGCTGCA |
| L2 L2S (10654-12958) | L2A           | AATGGCATGATATAATCTGACATCATATC |
| L3 L3S (12926-15461) | L3A           | ACCAAAACAAGAAGAAGACTCGTGTTGTAATG |
| 3’RACE (617) S       | S             | TTGAAATAGACGACAGACAGTGG |
| 3’RACE (399) Sn      | Sn            | GCGGATGAGGCTTCTTTACTTTATAC |
| 3’RACE (15088) S     | S             | CAGCGCGAGGCTTCTTTACTTTATAC |
| 3’RACE (15423) An    | An            | TGACATCGTTCATTACTCTTGTTG |

Additional material

Additional file 1: The detail recombination information of mosaic strain 81-19252_Texas-81_EU439429 (A, B) Results of Similarity and Bootscanning analysis of 81-19252_Texas-81_EU439429. The y-axis in Similarity plot (A) gives the percentage of identity within a sliding window of 500 bp wide centered on the position plotted, with a step size between plots of 20 bp, while in Bootscanning plot (B) represents the percentage of permuted trees. Shipping_Fever_AF178655 and 910N_DB4095 were used as two parental sequences and QS592_EU277658 an outgroup sequence. Four breakpoints were identified and located by GARD at position 8688, 12595, 13619 and 14175, respectively, with value maximized. The query sequence 81-19252_Texas-81_EU439429 demonstrated greater sequence identity and Bootscanning support with 910N_DB4095 in the second and fourth regions while otherwise with QS592_EU277658 in the complementary regions. (C-G) Neighbor-Joining Phylogenetic profiles of separate regions of 81-19252_Texas-81_EU439429 partitioned by crossover events. The scale corresponds to the number of nucleotide substitutions per site. The putative recombinants were showed with "black square". (C-G) represent the phylogeny of fir-(1-8688), sec-(8689-12595), thi-(12596-13619), fou-(13620-14175) and fin-(14176-15536) part of full length segment, respectively. The sec-and fou-part of mosaics demonstrated higher level of congruence with the 910N_DB4095 lineage, while the otherwise converge with Shipping_Fever_AF178655.

Additional file 2: The detail recombination information of mosaic strain 92-7783_ISU-92_EU439428 (A, B) Results of Similarity and Bootscanning analysis of 92-7783_ISU-92_EU439428. The y-axis in Similarity plot (A) gives the maximum percentage of identity within a sliding window of 500 bp wide centered on the position plotted, with a step size between plots of 20 bp, while in Bootscanning plot (B) represents the percentage of permuted trees. Shipping_Fever_AF178655 and...
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Authors' contributions

YHT, and JQ carried out sequence collection, alignment and recombination analysis and drafted the manuscript. ZX and BM provided L22 viral sequence information. SHL participated in sequence collection. HHB participated in its design and coordination. HCO designed the study and wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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