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Original Article

Characters of Yulong Yersinia pestis strains from Yunnan Province, China

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Abstract: In 2005, five human cases of primary pneumonic plague were reported in Yulong County in Yunnan province of China and resulted in two deaths. Several Yersinia pestis strains (designated Yulong strains) have been isolated from rats and fleas in this area since 2006, and a new plague focus in Yulong County has been confirmed. However, the source of these Yulong strains and their relationship with strains from nearby natural foci remain unclear. In this study we compared Yulong strains with previously isolated strains from Yunnan province by biochemical analysis, clustered regularly interspaced short palindromic repeats (CRISPR) and pulsed-field gel electrophoresis (PFGE) methods which revealed significant differences between the Yulong strains and early *Y. pestis* strains. However, the Yulong strains were found to be similar to Himalayan marmot strains. Therefore, Yulong strains are a new type of Y. pestis in Yunnan province and likely represent the first step in the transmission of Yersinia pestis from the Qinghai-Tibet Plateau to Yunnan province. The Yulong focus merits greater attention because of its special status of Yunnan plague epidemiology.

Keywords: Yersinia pestis, biochemical analysis, CRISPR, PFGE

Introdution

Plague is a natural focal disease. Two types of plague foci have been confirmed in Yunnan province of China: Rattus flavipectus plague focus (designated Focus A) and Apodemus chevrieri-Eothenomys miletus plague focus (designated Focus B). Focus A is known globally as the origin of the third plague pandemic [1] and is distributed mainly in the southern region of the Hengduan Mountains in the western, southwestern and southern areas of Yunnan province. This region locates at the wide valley or basin of a resident-culture landscape zone 400-2100 meters above sea level with a total area of 79,216 square kilometers. The main host of Yersinia pestis (Y. pestis) in focus A is R. flavipectus. There were 507 bubonic plague cases (two deaths) in focus A from 1982. Focus B was discovered in 1974 and distributed in the center of Jianchuan County in northwestern Yunnan province, which is located in pine forest and farmland landscape zones in mountain areas 2300-4000 meters above sea level with an area of approximately 1600 square kilometers. The main hosts in Focus B are A. chevrieri and E. miletus. No human case has been reported in Focus B.

Between Oct. 25 and Oct. 30 in 2005, five patients who travelled together in a car for approximately one hour presented high fever, cough with bloody sputum, and dyspnea in Luzi village of Huangshan town, Yulong County, Yunnan Province of China [2], two patients died due to severe illness at last. According to an indirect hemagglutination test (IHA), the sera collected from three convalescent patients at two weeks after the appearance of symptoms contained F1 antibodies at titers of 1:40, 1:80 and 1:160. However, all sera collected at one week after the appearance of symptoms were

negative for F1 antibodies. Based on epidemiological data and laboratory results, the five cases were diagnosed as pneumonic plague. This pneumonic plague epidemic event was caused by one primary pneumonic case through aerosol transmission.

The five pneumonic plague cases were first discovered in Yunnan province of China after the third plague pandemic. Yulong strains of Y. pestis may cause primary plague and are transmitted at a high rate. We have isolated Y. pestis at sites of primary pneumonic plague and obtained several strains of Y. pestis since 2006. Focus Yulong is connected to Focus B, and their habitats are similar. However, it is unclear to which type the Yulong strains belong, how they are related to the strains from the nearby natural foci and whether the Yulong strains are more virulent than adjacent foci strains.

Y. pestis strains can be differentiated by phenotypic and genotypic methods. Phenotype classification of Y. pestis, a classic method of Y. pestis typing, is based on glycerol, nitrate and arabinose fermentation [3]. There are several methods of molecular typing of *Y. pestis*, including pulsed-field gel electrophoresis (PFGE) [4], multi-locus variable-number tandem repeat (VNTR) analysis (MLVA) [5], clustered regularly interspaced short palindromic repeats (CRISPR) analysis [6], different region (DFR) analysis [7], SNP genotyping [1], and IS*100* insertion element RFLP analysis [8].

The Y. pestis genome contains three CRISPR loci: YPa (YP1), YPb (YP2) and YPc (YP3). More than 150 spacer sequences have been reported [9]. The distributions of spacers and their arrays in Y. pestis strains are significantly region- and focus-specific, enabling the construction of a hypothetic evolutionary model of Y. pestis. PFGE is the gold standard of bacterial molecular typing techniques. The PulseNet network, which is based on PFGE analysis, plays an important role in tracing the origin of disease [10]. Gene rearrangement is common in Y. pestis due to large number of insertion sequences (IS) in its genome, as confirmed by PFGE analysis [11, 12]. In this study, we used biochemical identification, CRISPR and PFGE typing analysis to compare Yulong *Y. pestis* strains with the strains isolated from Focus A and Focus B.

Materials and methods

Strains

In this study, we analyzed 30 Y. pestis strains, including the vaccine strain EV76 and 29 Y. pestis strains isolated from Focus A, Focus B and the Yulong plague focus between 1964 and 2007 from infected humans, animals, and fleas in Yunnan province, China. Among the Yunnan strains, a total of 24 Y. pestis strains were isolated from Focus A (six strains isolated from Lianghe County, seven strains isolated from Yingjiang County, four strains isolated from Lingcang County, six strains isolated from Wenshang County and one strain isolated from Jinghong County). Two strains were isolated from Focus B in Jianchuan County, and three Yulong strains were isolated from Yulong focus in Yulong County. These strains are listed in **Figure 1** and were obtained from the Yunnan Institute for Endemic Diseases Control and Prevention (YIEDC).

Culture media for determining biochemical characteristics

Culture media for determining biochemical characteristics were prepared as previously described [12]. In brief, sugar-free semisolid culture media with 1.6% BTB indicator and supplemented with 1% rhamnose, maltose, sucrose, mannitol, arabinose, glucose, melezitose, melibiose, salicin, lactose, dextrin or glycerol were used for sugar fermentation. Then, 3 ml of media was aliquoted into test tubes and sterilized with 8 pounds of pressure for 15 minutes. Media for nitrification and denitrification tests, methyl red test, and VP tests were also prepared to identify the above strains. These media were autoclaved and stored overnight at 37°C prior to sterility tests.

Determination of biochemical characteristics

Y. pestis strains inoculated on the slants were cultured at 28°C for 24 h. Bacteria were inoculated into the media described above using a disposable inoculating loop. For the fermentation test, the bacterial cultures were incubated at 37°C for 7 days followed by 7 days at room temperature and observed once every two days. For the nitrification and denitrification tests, bacteria inoculated into the appropriate media were cultured at 28°C for 2 days. Then,

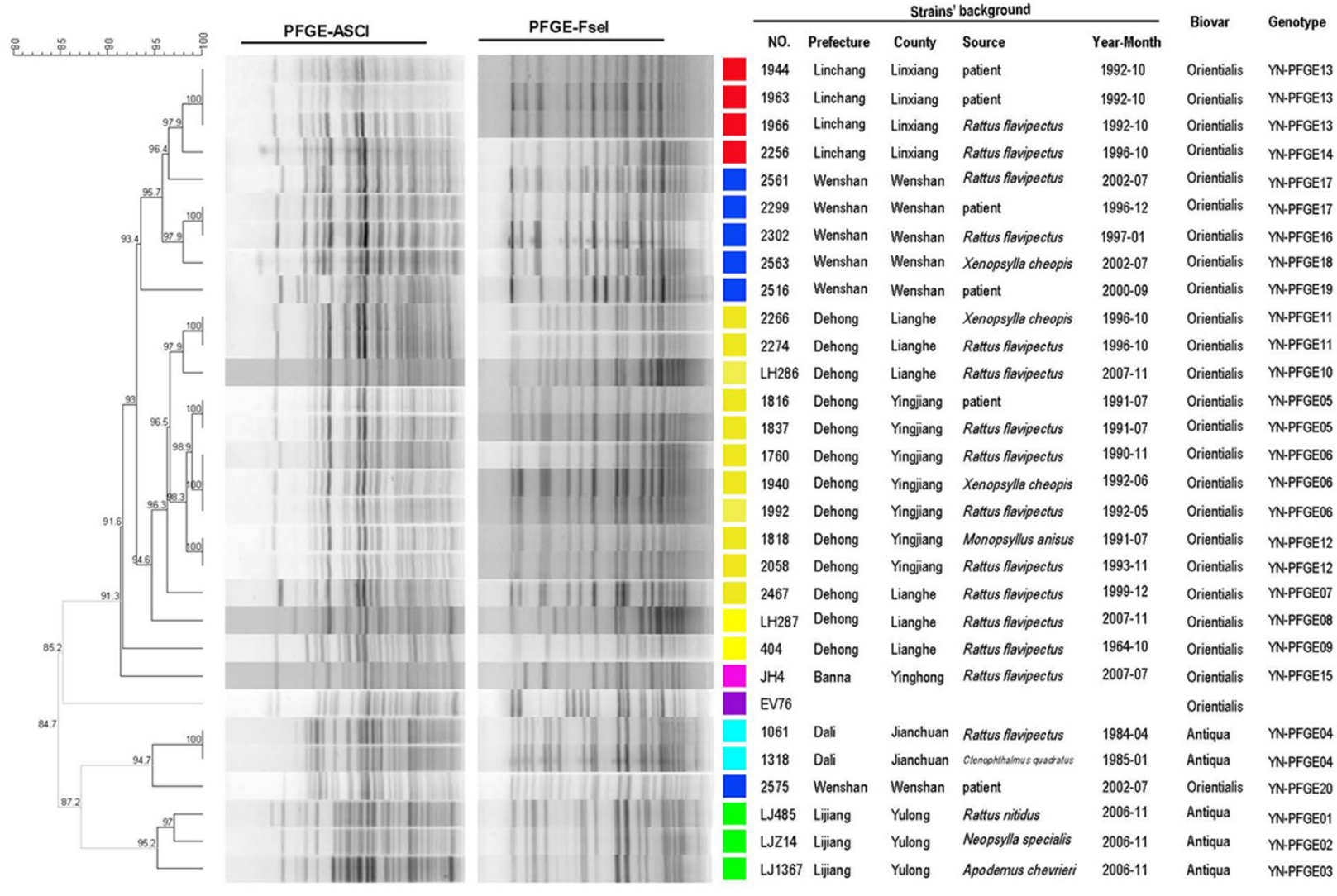

Figure 1. PFGE profiles of the Y. pestis strains isolated from Yunnan. The cluster diagram for each vertex of a cluster is dashed.

Table 1. Biochemical characteristics of the Y. pestis strains from Yunnan

Strains	Malt-ose	Glyc-erol	Sali-cin	Dex-trin	Denitrifi-cation	Rhamnose sugar	Su-crose	Arabi-nose	Man-nitol	Glu-cose	Melezi-tose	Disac-charide	Lac-tose	Denitrifi-cation	Nitrifica-tion	Methyl red	VP
Focus A Strains (24)	+	-	+	+	+	-	-	+	+	+	-	-	-	+	-	+	-
Focus B Strains (2)	-	+	+	-	+	-	-	+	+	+	-	-	-	+	-	+	-
Focus Yulong Strains (3)	+	+	-	+	+	-	-	+	+	+	-	-	-	+	-	+	-
EV76	+	-	+	+	+	-	-	+	+	+	-	-	-	+	+	+	-

the cultures were analyzed by adding Gliss solution. For the methyl red and VP tests, bacteria inoculated into appropriate media were cultured at 28°C for 4 days. Then, the cultures were mixed with several drops of 0.02% liquid methyl red ethanol for the methyl red test or an equal volume of 10% KOH for the VP test. The mixtures were allowed to stand for one day before observing the reactions.

CRISPR loci amplification and sequencing analysis

Three CRISPR loci (YPa, YPb and YPc) were amplified by PCR using the following primer pairs targeting the region flanking the CRISPR [13]: YP1-L (5'-AATTTTGCTCCCCAAATAGCAT-3') and YP1-R (5'-TTTTCCCCATTAGCGAAATAAGTA-3') for the YPa region; YP2-L (5'-ATATCCTGCTTACCGAGGGT-3') and YP2-R (5'-AATCAGCCACGCTCT GTCTA-3') for YPb; and YP3-L (5'-GCCAAGGGATTAGTGAGTTAA-3') and YP3-R (5'-TTTACGCATTTTGCGCCATTG-3') for YPc. PCR was performed in a volume of 30 ml containing 1 μl of DNA template, 0.5 μl (10 μmol/L) of each primer, 15 ml of PCR reaction mixture (Premix Ex Taq) and 13 ml of ddH_2O. The cycling conditions were 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 40 s, annealing at 58°C for 40 s, extension at 72°C for 1 min, and a final extension at 72°C for 5 min. The PCR products were sequenced, and the space sequences were identified using "CRISPRs Finder" software available online (http://crispr.u-psud.fr/). The gene cluster and strain types were determined with the method of Cui [6].

PFGE

Genomic DNA from the Y. pestis strains was prepared in agarose plugs as described previously [14], with some modifications. Briefly, Y. pestis strains were cultured in BL21 medium at 28°C for 24 h. The Y. pestis cells were then collected by centrifugation and suspended in lysis buffer (10% SDS, pH 8.0) containing lysozyme (0.1 mg/ml). The suspension was mixed with an equal volume of 1.6% PFGE-grade melted agarose (Bio-Rad Laboratories, Richmond, Calif.), poured into plug molds (catalogue no., 1703706; Bio-Rad Laboratories), and incubated at 4°C for 30 min. The plugs were placed into sterile 50-ml blue-cap tubes and digested with 1 mg/ml proteinase K. Protein digestion was terminated by adding phenylmethylsulfonyl fluoride (0.5 mg/ml). The plugs were washed with 1×TBE (90 mM Tris-borate, 2 mM EDTA) before use and digested overnight at 37°C with the endonucleases AscI and FseI respectively. Finally, the restriction fragments were resolved by PFGE with a CHEF Mapper apparatus (Bio-Rad Laboratories) in 1×TBE in 1% agarose gels at 200 V. The gels were maintained at 14°C during electrophoresis. The pulse times were ramped from 2 to 25 s over 20 h at 20 V/cm. The profile was analyzed using the Dice cluster analysis method in BioNumerics (Version 6.6, Applied Maths Company, Kortrijk, Belgium). The Dice coefficient was used to analyze the similarities of the banding patterns. The unweighted pair group method with average linkages (UPGAMA) was used for cluster analysis. The cluster cutoff value was automatically defined by the software ''clusters''. Finally, the corresponding PFGE genotypes were marked in each locus of Yunnan province with arcGIS10.1 software (Esri corporation, New York, USA).

Results

Biochemical characteristics of Y. pestis strains from Yunnan province, China

The biochemical characteristics of the strains isolated from the same foci were identical. Strains from different foci differed in terms of maltose, salicin, dextrin and glycerol fermentation (see **Table 1**). The strains from Focus A and Yulong County were able to ferment maltose and dextrin, while the strains from focus B were dextrin negative. The strains from Focus B and Yulong County were able to ferment glycerol, while the strains from Focus A could not. The strains from Focus A and B were able to use salicin, while the strains from Yulong were salicin negative.

CRISPR typing of Y. pestis strains from Yunnan province, China

In accordance with the CRISPR genotyping standard, the 30 strains of Y. pestis were classified into three genotypes belonging to three gene clusters: genotype 35 of gene cluster Ca52, genotype 30 of gene cluster Ca8, and genotype 22 of gene cluster Ca7 (see **Table 2**). Two strains from Focus B (Jianchuan County) were genotype 35 of gene cluster Ca52. Three Yulong Y. pestis strains were genotype 22 of

Table 2. CRISPR typing results of the Y. pestis strains from Yunnan province, China

	No. of strains	Cluster	Genotype	Spaces		
				Ypa	Ypb	Ypc
Focus A Strains	24	Ca8	30	a1-a2-a3-a4-a5-a6-a7-a8	b1-b2-b3-b4-b5	c1-c2-c3
Focus B Strains	2	Ca52	35	a1-a2-a3-a5-a6-a7-a52	b1-b2-b3-b4	c1-c2-c3
Focus Yulong Strains	3	Ca7	22	a1-a2-a3-a4-a5-a6-a7	b1-b2-b3-b4	c1-c2-c3
EV76	1	Ca8	30	a1-a2-a3-a4-a5-a6-a7-a8	b1-b2-b3-b4-b5	c1-c2-c3

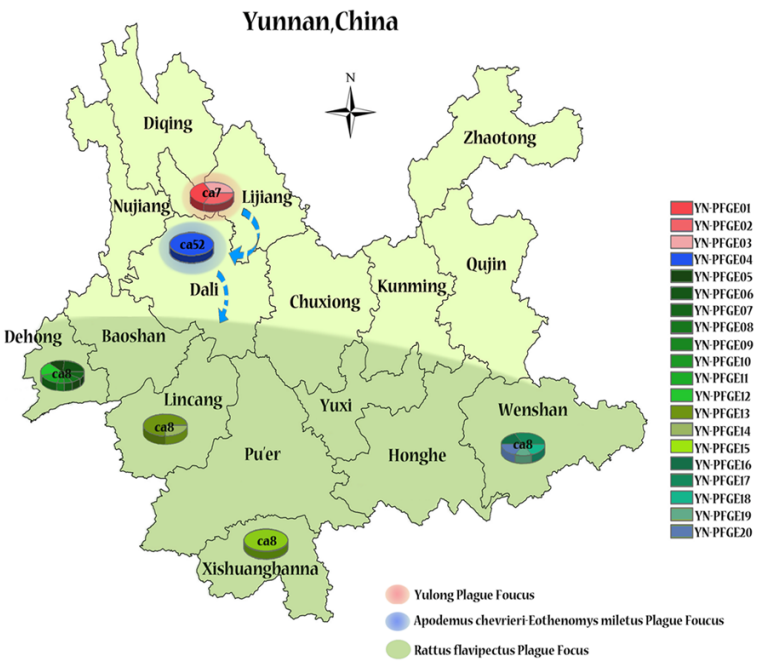

Figure 2. The geographic distribution of different Y. pestis strains classified by CRISPR and PFGE genotypes in Yunnan Province.

gene cluster Ca7. Twenty-four strains from Focus A and vaccine strain EV76 were genotype 30 of gene cluster Ca8.

PFGE typing of Y. pestis strains from Yunnan province, China

The Y. pestis strains from Yunnan were classified into 20 PFGE genotypes belonging to the three major clusters by digestion with *Asc*I and *Fse*I (see **Figure 1**). The three major PFGE clusters were the Yulong gene cluster, Jianchuan gene cluster and R. flavipectus gene cluster. The PFGE profiles of the three Yulong strains exhibited 95% similarity. The similarity between the PFGE profiles of the Yulong and Jianchuan gene clusters was 87.2%, while the similarity between the Yulong and *R.* flavipectus gene clusters was 84.7%.

The PFGE profiles of two strains in the Jianchuan gene cluster were identical. A strain isolated from one patient in Wenshan County (strain No. 2575) that belonged to Focus A was classified into Jianchuan gene cluster. This strain was also highly similar (95%) to the PFGE profiles of the two strains isolated from Jiangchuang County.

The R. flavipectus gene cluster can be divided into three sub-clusters: Dehong, Wenshan and Lincang. All Y. pestis strain isolated from Dehong belonged to the Dehong subcluster, and most of were greater than 96% similar to the PFGE profiles, except for two strains: No. 404 and No. LH287.

In addition, as a control, vaccine strain EV76 was classified as a separate PFGE genotype. Although the EV76 and *R.* flavipectus strains were both biovar Orientalis, the similarity of the PFGE gene cluster between the EV76 and R. flavipectus strains was 85.2%, and the similarity among the EV76, Jianchuan and Yulong gene clusters was 84.7%.

Geographic distribution of the CRISPR and PFGE genotypes

The Y. pestis strains isolated from Focus A, Focus B and Yulong in Yunnan had unique genotypes. Each genotype had its own geographical distribution, without any crossover (**Figure 2**). Three new strains isolated from the Yulong plague focus were Ca7 CRISPR type. PFGE typing indicated that these strains belonged to the Yulong gene cluster and could be classified into three genotypes: YN-PFGE01, YN-PFGE02 and YN-PFGE03. Two strains isolated from Focus B were Ca52 CRISPR type and belonged to the PFGE type designated

as YN-PFGE04. Twenty-four strains isolated from Focus A were Ca8 CRISPR type and included 15 PFGE types. YN-PFGE13 was the major genotype in Lincang prefecture; YN-PFGE17 was the major genotype in Wenshan prefecture; YN-PFGE06, YN-PFGE05 and YN-PFGE12 were the major genotypes in Yingjiang County, Dehong prefecture; and YN-PFGE11 was the major genotype in Lianghe County, Dehong prefecture, Yunnan province.

Discussion

Y. pestis strains isolated from Yulong County are a unique ecological type in Yunnan

Different ecological types of Y. pestis have specific geographic distributions, which are closely associated with the composition of biological communities and the physiological and biochemical characteristics of the primary host animals and vector insects as a result of long-term co-evolution and natural selection [15]. The Yulong focus is located in the middle of the Hengduan mountain (2300-4000 meters above sea level). The mountain vegetation is a mixture of oak trees, pine trees and brushwood. The mountain basin is composed of farmland on the border of plague Focus B (Jianchuan focus). A. chevrieri and E. miletus are the dominant hosts of the plague pathogen in this region. Neopsylla specialis and Ctenophthalmus quadratus are the dominant fleas serving as major vectors. Therefore, the Yulong focus is generally consistent with the Jianchuan focus in terms of the characteristics of the vegetation, natural landscape, rodents and fleas. However, Yulong strains have certain differences compared with Jianchuan strains, according to an analysis of their biochemical characteristics. Maltose fermentation is an important marker for biochemical identification of Y. pestis; Yulong strains can ferment maltose, while Jianchuang strains cannot. Note that the biochemical characteristics of Yulong strains are identical to those of strains isolated from Himalayan marmot in Qinghai, China. A branch of Y. pestis in China may have been transmitted from the Tian Shan Mountains to northwestern Yunnan through the Pamirs Plateau and the Qinghai Tibet Plateau, finally arriving in the Guangdong and Yunnan region [16]. Thus, it is possible that Yulong strains are an important link to plague distribution in western China, that is, the Yulong plague evolved from marmot plague and then spread to Jianchuan County to form Focus B (Jiangchuan plague focus), which spread to southern Yunnan to form Focus A (Yunnan R. flavipectus plague).

Yulong strains are an ancient genotype in Yunnan

According to the CRISPR site evolution model, the strains of the three plague foci in Yunnan province, China, may have evolved from the same ancestor strains. The Yulong strain of Y. pestis belong to the Ca7 CRISPR gene cluster, which represents earlier stages of CRISPR sequence evolution in Yunnan Y. pestis strains. Beginning in Tajikistan (subsp. hissarica), one branch of the plague transmission route of bv. microtus in China passed through the Kunlun Mountains and to the Qinghai-Tibet Plateau before transmission to Yunnan province and other locations throughout the world through human activity [7]. Therefore, the Yulong strains are the first stop in the transmission of *Y. pestis* from the Qinghai-Tibet Plateau to Yunnan.

Thus, the Yulong strains are the most ancient strains in Yunnan province. Strains of Focus B belong to the Ca52 CRISPR gene cluster, which evolved from Yulong strains in Yunnan by randomly losing region sequences a4 and polarly inserting a52 at the YPa site. Strains of Focus A evolved from Yulong strains in Yunnan by polarly inserting a8 at the YPa site and b5 at the YPb site, which is the CRISPR genotype 30 of the Ca52 gene cluster.

Yulong strains are independent PFGE genotypes in Yunnan

In this study, we analyzed the different ecological type strains from different plague foci in Yunnan province, China. Our results indicated that the PFGE types of the strains were consistent with the ecotypes of the corresponding strains. The ecotypes of the R. flavipectus plague pathogen, A. chevrieri and E. miletus strains and Yulong strains were classified into three corresponding gene clusters, and PFGE typing disclosed the inherent differences among these strains. Cluster typing of the PFGE types revealed greater polymorphism and useful information than traditional ecological classification, in addition to shedding light on the genetic characteristics of Y. pestis evolution. Our PFGE typing indicated that Yulong strains

differed from the R. flavipectus strains and Jianchuan A. chevrieri and E. miletus strains, while Yulong strains were an independent gene cluster. Our results are also consistent with the MLVA typing findings from a CDC research group in China [16].

Possible explanation for some special strains

Among the Y. pestis strains isolated from Dehong state, the genotypes of strain No. 404 and LH287 are significantly distinct from those of other strains. Strain 404, a late strain that caused the last major plague pandemic in Yunnan, was isolated in 1964. Strain LH287, which was also a late strain during a period of R. flavipectus plague recrudescence in Yunnan province from 1982 to 2007, was isolated in 2007. Thus, it was presumed that 2007 was the last year of this pandemic. We observed a great difference between the pandemic Y. pestis strain isolated in the mid-20 century and the pandemic Y. pestis strain recrudescing among R. flavipectus in 1982 in Yunnan province. The reasons for this difference are unclear and require further study.

Conclusions

In summary, based on phenotype or genotype, Yulong Y. pestis strains are a unique type of Yersinia pestis that differ from those found earlier at other sites in Yunnan province, China. Yulong Y. pestis strains are more likely to have evolved from strains associated with Himalayan marmot in Qinghai, China. Given the potential risks of an epidemic of pneumonic plague, this new natural focus merits greater attention and the development of specific strategies to guide plague prevention and control.

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Disclosure of conflict of interest

None.

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