Original Article

Phylogenetic analysis of clinical strains of *Helicobacter pylori* isolated from patients with gastric diseases in Tibet

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Background: *Helicobacter pylori* (*H. pylori*) is a Gram-negative pathogenic bacterium that causes chronic gastritis and other gastric diseases in humans. In Tibet, China, the infection of *H. pylori* is an important risk factor that caused gastric cancer.

Methods: To understand the characteristics of this pathogen in Tibet, five strains of *H. pylori* were isolated from three patients' oral cavity or stomach who had either a gastric ulcer or gastritis. We performed genome sequences of these five clinical strains on Illumina Hiseq, and 55,016-63666 SNVs/InDels were identified by comparing to the reference strain of *H. pylori* 26995.

Results: The phylogenetic analysis with multi-locus sequence typing (MLST) showed that five Tibetan strains were defined as hpEurope population and their proteins encoded by the *cagA* gene also presented a western type. Also, the strains that were isolated from the same patients' oral cavity and stomach exhibited homology in molecular evolution.

Conclusions: This is the first study to investigate the phylogenetic population structure of the epidemic strains of *H. pylori* in Tibet, which may improve cognition of Tibetan strains and confirm the homology of the strains from oral cavity and stomach.

Keywords: *Helicobacter pylori* (*H. pylori*); Tibet; oral strain; multi locus sequence typing

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Introduction

Helicobacter pylori (*H. pylori*) is a Gram-negative microaerophilic gastric pathogen, which could colonize in the upper gastrointestinal tracts over 50% population of the world (1). In 1982, *H. pylori* were isolated from gastric mucosa of a patient with chronic gastritis in Australia for the first time (2,3). Over the past three decades, a large number of studies on *H. pylori* have been carried out. It has shown that the infection of *H. pylori* is one of the major pathogenic reasons of chronic gastritis, peptic ulcer, gastric mucosa-associated lymphoid tissue (MALT) lymphoma and gastric cancer (4-6). The International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) has defined *H. pylori* as class I carcinogen. Also, *H. pylori* can take up residence in the oral cavity and has been widely isolated from oral mucosa, plaque, and saliva of the oral disease's sufferers (7,8). Oral *H. pylori* strains are

associated with dental caries, periodontitis, mouth ulcers, ozostomia, and other oral diseases. *H. pylori* that were living in the oral cavity can be swallowed with saliva or food into the stomach, and the pathogen also can be vomited into the mouth from the stomach. However, the study on the oral *H. pylori* is scarce and far less than isolates from the stomach. The differences between the *H. pylori* strains that were isolated from the stomach and oral cavity are still controversial.

The initial infection of *H. pylori* usually occurs in childhood and always presents family aggregation (1,9,10). Without antibiotic eradication therapy, the infection of *H. pylori* in adults tends to be persistent lifelong. Many studies have shown that the prevalence of the infection of *H. pylori* was positively correlated with age (11,12). It is reported that the incidence and prevalence of the infection of *H. pylori* are common in China, especially in Tibet (11,13). An epidemiological investigation in 2010 (14) showed that the total infection rate of *H. pylori* in Chinese natural population was 56.22%, and it also showed significant differences among different areas of China. The lowest infection rate was 42.01% in Guangdong Province, while the highest was 84.62% in the Tibet Autonomous Region. It is noteworthy that Tibet is a high incidence of gastric cancer mortality area (13). According to the first gastric cancer census of Tibet in the 1970s, it suggested that the highest mortality of malignant tumors was gastric cancer, which accounted for 48.89% of total fatality of malignant tumors. Since then, gastric cancer mortality in Tibet has remained high always caused most cancer deaths despite a slight decline (15,16). Although backward economic development and poor hygiene conditions are confounding factors for *H. pylori* infection, the *H. pylori* strain epidemic in Tibet may present high pathogenicity and carcinogenicity.

Multi-locus sequence typing (MLST) is a rapid and effective method that based on allelic variation among several conserved housekeeping genes and has been applied to categorize and trace epidemic isolates of many pathogenic bacteria species for nearly two decades. In 2003, an MLST protocol for *H. pylori* employing seven housekeeping genes (*ureI*, *mutY*, *EFP*, *PPA*, *ypbC*, *atpA*, and *trpC*) and one virulence-associated gene (*vacA*) was established, and this protocol divided 370 *H. pylori* strains all over the world into seven populations and subpopulations on the basis of their geographical distribution (17). In recent years, next generation sequencing (NGS) has been widely used in studying the genomics of bacteria and can provide much more detailed and precise genetic information (18). Until now, a large number of MLST typing and genome sequences of *H. pylori* strains have been obtained and published on a public database. However, there is only one *H. pylori* strain that isolated from a Tibetan patient with gastric cancer and was conducted genome sequencing. The similarity to the reference strain *H. pylori* 26695 was only 87.25% (16).

Tibet Autonomous Region is located in the Qinghai-Tibet Plateau, where the average elevation is over 4,000 meters. Unique geographical environment and weather conditions created a series of unique species distributions. The strain of *H. pylori* epidemic in Tibet may be more pathogenic and carcinogenic to maintain the high incidence of *H. pylori* infection and gastric cancer.

In this work, we intended to study the characteristics and structure of the epidemic strains of *H. pylori* in Tibet. To achieve this purpose, five *H. pylori* clinical strains in Tibet were isolated, identified and performed the genome sequencing. By comparing the genomic sequences of *H. pylori* 26695, the single nucleotide variants (SNVs) and Insertion-Deletions (InDels) were analyzed to learn more specificity of Tibetan strains. Also, 76 worldwide strains were performed phylogenetic analysis to study bacterial relationship and explore the population structure of the five epidemic strains of *H. pylori* in Tibet.

Methods

Isolation, culture, and identification of H. pylori strains

The study participants were recruited in gastroscopy room of Hospital of Chengdu Office, People's Government of Tibet Autonomous Region during the period from December 2015 to February 2016. All participants did not have systemic diseases, and no antibiotics were used in one week before gastroscopy. Gastric mucosal tissues and saliva samples were collected for isolation of *H. pylori* clinical strains. Briefly, the gastric mucosal and salivary samples were tested by Urease Test. The positive samples were subsequently cultured on Columbia agar plates (Land Bridge, Beijing, China) with 5% sheep blood, at 37 ℃ in a microaerophilic environment containing 5% O_2, 10% CO_2, and 85% N_2 for 72 h. After colonies appeared, gram stain and microscopic examination were carried out for initial identification; the bacterial colony was confirmed by a series of biochemical tests, such as Catalase, Oxidase and Urease Tests for further identification. *H. pylori* animal type strain, Sydney Strain 1 (SS1), was also cultured on Columbia agar

Table 1 Origin and clinical background of *H. pylori* strains that were isolated from Tibet

Strain ID	Source	Patient ID	Gender	Age (years)	Race	Diseases
XZCB-W009	Stomach	9	Male	30	Tibetan	Gastric ulcer
XZCB-K00901	Oral cavity	9	Male	30	Tibetan	Gastric ulcer
XZCB-K00902	Oral cavity	9	Male	30	Tibetan	Gastric ulcer
XZCB-K002	Stomach	2	Female	74	Han	Gastritis
XZCB-K038	Stomach	38	Female	75	Tibetan	Gastritis

base with 5% sheep blood as described above.

Five clinical strains were isolated from three patients with gastric diseases (*Table 1*). Of these, *H. pylori* XZCB-W009, XZCB-K00901 and XZCB-K00902 were isolated from patient No. 9, a 30-year-old male Tibetan patient with gastric ulcer, as XZCB-W009 was isolated from the stomach, XZCB-K00901 and XZCB-K00902 were from the oral cavity. XZCB-K002 was isolated from the stomach of patient No. 2, a 74-year-old female Han patient living in Tibet with gastritis. XZCB-K038 was isolated from the stomach of patient No. 38, a 75-year-old female patient with gastritis.

DNA extraction and Illumina sequencing

H. pylori strains were harvested from Columbia agar plates for DNA extraction, which was carried out by using TIANamp Bacteria DNA Kit (TIANGEN, Beijing, China) according to manufacturer's instructions. The genomic DNA was then sequenced on a sequencer of Illumina Hiseq (2×150 bp) at GENEWIZ Inc. (Suzhou, China). The low quality reads were filtered by using Trimmomatic (v 0.30) (19) and Cutadapt (v 1.9.1) (20). The clean data were aligned to the reference genome of sequenced strain *H. pylori* 26695 (Accession Number NC_CP010436.1) by using BWA (v 0.7.12) (21). Identification of SNVs/InDels for each base site was performed by using SAMSTOOL (v 1.1) (22). The Annovar software was used for functional annotation on gene loci variation (23).

The genome sequences of these five Tibetan strains reported in this paper have been uploaded to the NCBI Sequence Read Archive (SRA) database under the BioProject SRP127752 with BioSample accession number SRS2800546, SRS2800545, SRS2800544, SRS2800548, and SRS2800547. The datasets generated during and analyzed during the current study are available in the SRA repository on the website of www.ncbi.nlm.nih.gov/sra/?term=SRP127752.

MLST analysis

For the MLST analysis, seven housekeeping genes (*ureI*, *mutY*, *EFP*, *PPA*, *yphC*, *atpA*, and *trpC*) and one virulence-associated gene (*vacA*) of five *H. pylori* strain from Tibet were selected and recombined as previously described (17,24). To analyze the phylogeny of the Tibet strains, eight gene fragments of the 76 *H. pylori* strains that isolated from 28 different countries across the five continents were downloaded from MLST database http://www.mlst.net (*Table 2*). Next, the ClustalX (v 1.81) was used for multiple sequence alignment of MLST genes (25). The alignments were used for phylogenic analysis with MEGA (v6.06) (26). Distance-based phylogenetic tree for MLST genes was calculated by using the Tamura-Nei model, and Bootstrap analysis was performed with 1,000 replications.

cagA gene

The *cagA* genes of five Tibetan strains were extracted from the genome sequence for phylogenetic analysis with strain 26695 and then were translated into protein sequence for EPIYA motifs detection.

Negative stain and electron microscope

H. pylori SS1, XZCB-W009, and XZCB-K00901 were cultured on Columbia agar plates for 72 h. The bacteria were suspended in 2% ammonium acetate to remove the medium, and stained by 2% sodium phosphotungstate. The samples were applied to copper Formvar-coated grids and observed under JEM-ARM 200F (JEOL) transmission electron microscope (TEM).

Table 2 Geography and structure of strains for phylogenetic analysis used in this study

Isolates	Area	Population	Isolates	Country/area	Population
BJ413	Beijing	ND	y1	Russia	hpEurope
BJ432	Beijing	ND	y12	Russia	hpEurope
ccs9	Chongqing	ND	y4	Russia	hpEurope
xinan4	Chongqing	ND	bo265	Germany	hpEurope
guang1	Guangzhou	ND	ku204	Germany	hpEurope
guang4	Guangzhou	ND	be158	Italy	hpEurope
hang268	Hangzhou	ND	be194	Italy	hpEurope
hlj193	Heilongjiang	ND	10CH	Spain	hpEurope
hlj200	Heilongjiang	ND	11CH	Spain	hpEurope
HB448	Hubei	ND	Fr-B41-M	France	hpWAfrica
HB460	Hubei	ND	Fr-G12-G	France	hpEurope
HBKTN2	Shijiazhuang	ND	nl586	Netherlands	hpEurope
HBKTN3	Shijiazhuang	ND	nl600	Netherlands	hpEurope
xa57	Xi'an	ND	26695	UK	hpEurope
yn32	Yunnan	ND	001uk	UK	hpEurope
yn326	Yunnan	ND	fi106	Finland	hpEurope
wls-5-3	Zhejiang	ND	Sw-C520-G	Sweden	hpEurope
wls-5-7	Zhejiang	ND	Sw-C577-G	Sweden	hpEurope
HK182	Hong Kong	hspEAsia	inma10	Canada	hspAmerind
HK35	Hong Kong	hspEAsia	j166	USA	hpEurope
Tw109Pa	Taiwan	hspMaori	j99	USA	hspWAfrica
TwM4	Taiwan	hspEAsia	lsu1010-1	USA	hpEurope
K15	Japan	ND	nq1677	Colombia	hpEurope
K16	Japan	ND	nq1725	Colombia	hpEurope
DU15	Korea	hspEAsia	ausabrJ29	Australia	hpEurope
DU2	Korea	hspEAsia	auseurP156	Australia	hpEurope
CAM1	Cambodia	hpEurope	auseurP176	Australia	hpEurope
CAM4	Cambodia	hspEAsia	auseurP209	Australia	hpEurope
re03027	Malaysia	hpAsia2	NCTC11637	Australia	hpEurope
re03028	Malaysia	hspEAsia	NCTC11638	Australia	hpEurope
re7006	Singapore	hpEurope	SS1	Australia	hpEurope
re8033	Singapore	hpEurope	ne600	New Zealand	hspMaori
VIE2759	Vietnam	hspEAsia	ne605	New Zealand	hpEurope
VIE2770	Vietnam	hspEAsia	ne614	New Zealand	hpEurope
L113	India	hpEurope	ETH28	Ethiopia	hpNEAfrica
L122	India	hpEurope	ETH29	Ethiopia	hpNEAfrica
tur3321	Turkey	hpEurope	137	South Africa	hspSAfrica
tur673	Turkey	hpEurope	D1a	Senegal	hspWAfrica

Table 3 Description of SNVs/InDels among five Tibetan strains by comparing to the reference genome of *H. pylori* 26695

Strain ID	SNVs & InDels	SNVs in coding region				InDels in coding region				
		Non-synonymous	Synonymous	Stop codon mutation	Total	Frame shift insertion	Non-frame shift insertion	Frame shift deletion	Non-frame shift deletion	Total
XZCB-W009	55,033 & 73	14,971	33,830	117	48,918	4	4	6	6	20
XZCB-K00901	55,045 & 71	14,983	33,815	121	48,919	6	2	4	5	17
XZCB-K00902	54,929 & 87	14,951	33,786	119	48,856	7	4	7	5	23
XZCB-K002	63,504 & 162	18,277	38,475	148	56,900	15	9	8	8	40
XZCB-K038	63,532 & 122	18,311	38,456	149	56,916	11	5	2	4	22

Results

Characteristics of H. pylori clinical strains

Gram stain and a series of biochemical tests were used for identification of *H. pylori* clinical strains. The *H. pylori* strains showed seagull or spiral shape under the microscope (data not shown). In biochemical tests, all the strains expressed positively for catalase, oxidase and urease tests (data not shown).

SNVs/InDels

In order to identify the genic mutations of the *H. pylori* strains from Tibet, genomes of five strains were sequenced via Illumina Hiseq platform. The sequencing harvested a 700× average depth and a >92% breadth coverage of the reference genome.

Genotypes of loci were ascertained by using the Bayesian algorithm to calculate the posterior probability of loci. Based on the differences between the calculated genetic loci and the reference genome of *H. pylori* 26695, each different genetic locus was predicated as SNVs or InDels. Among the five *H. pylori* clinical strains, 55,016–63,666 SNVs/InDels were detected and identified. Of which, there were approximately 89% of the SNVs/InDels located in the coding regions, and the remains were located in the non-coding regions, the downstream region and the intersection area of one upstream region and the other downstream region. It could be found that the genome sequences of all *H. pylori* strains were significantly different from the reference strain. The details of SNVs/InDels were shown in *Table 3*.

The SNVs located in the coding region were calculated and analyzed. Based on the functional alterations of the triplet codes, the SNVs were divided into three groups: synonymous mutation, non-synonymous mutation and stop codon mutation (referring to a stop codon mutates into a codon that encodes an amino acid, or an amino acid-encoding codon mutates into a stop codon). It was found that there were 33,786–38,475 and 14,951–18,311 SNVs in the synonymous group and non-synonymous group, accounted for 67.6–69.5% and 30.3–32.2% of the total SNVs numbers, respectively. Also, 116–149 SNVs, less than 0.3% of the total, were in stop codon mutation group (*Table 3*).

Based on breadth alteration of the open reading frames (ORFs) attributed to the insertion or deletion of the sequence fragments, the InDels in coding region were divided into four groups: non-frame shift insertion, frameshift insertion, non-frame shift deletion, and frameshift deletion. There were 17–40 InDels in the *H. pylori* clinical and type strains, comprising 2–9 non-frame shift insertions, 4–15 frameshift insertions, 4–13 non-frame shift deletions and 2–8 frameshift deletions (*Table 3*).

MLST analysis

Fragments of seven house-keeping genes and one virulence-associated gene of each *H. pylori* strain were extracted and recombined. Among the 3,850 nucleotides, 1,250 were polymorphic and were used for phylogenetic analyses. Maximum likelihood unrooted tree diagram by MLST (*Figure 1*) showed that a total of 81 isolates were assigned into four groups: Asia, Oceania, Europe, and Africa group. Based on the molecular evolutionary relationship, all these isolates are defined by geography position and existing population.

H. pylori strain with defined Subpopulations hspEAsia, hpAsia 2, hspMario and hspAmerind were classified into Asia group. There were 18 strains that isolated from Beijing,

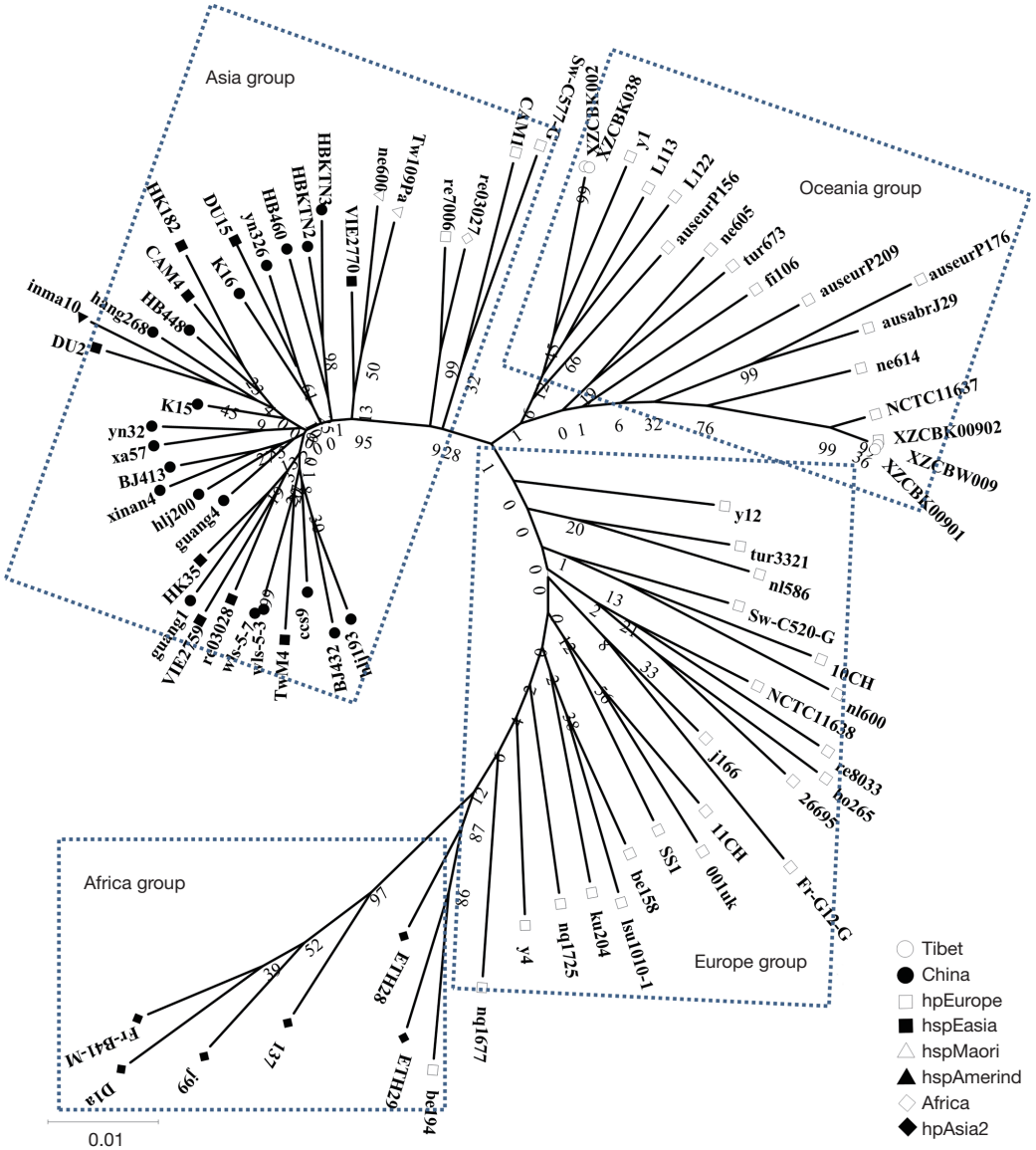

Figure 1 Phylogenetic analysis of 5 Tibetan and 76 worldwide *H. pylori* strains by using Multi Locus Sequence Typing method with seven housekeeping genes (*ureI*, *mutY*, *efp*, *ppa*, *yphC*, *atpA* and *trpC*) and one virulence-associated gene (*vacA*). All strains were divided into four group, Asia, Oceania, Europe and Africa group by geography. Five Tibetan strains were labeled with ○. Isolates without population definition from ten cities or areas in China, Beijing, Chongqing, Guangzhou, Hangzhou, Heilongjiang, Hubei, Shijiazhuang, Xi'an, Yunnan and Zhejiang, were labeled with ●. Population hspWAfrica, hspSAfrica and hspNEfrica were assigned into Africa with a label of ◇. The hpEurope, hspEsia, hspMaori, hspAmerind and hpAsia2 were labeled with □, ■, Δ, ▲ and ◆, respectively.

Chongqing, Guangzhou, Hangzhou, Heilongjiang, Hubei, Shijiazhuang, Xi'an and Zhejiang of China, and two strains from Japan without a definition of structure population or subpopulation, which were assigned into Asia group. However, there were two strains isolated from Malaysia and Cambodia, which were identified as hpEurope. Another hpEurope strain from Sweden was located on a small branch next to the centric node in the Asia group.

A majority of isolates from Australia and New Zealand were assigned into Oceania group, an hpEurope population cluster. Interestingly, five Tibetan isolates were also classified in this cluster; *H. pylori* XZCB-K002 from a Han

in Tibet and XZCB-K038 from a Tibetan were clustered in one branch with a Russian strain y1, regardless of race. XZCB-W009, XZCB-K00901, and XZCB-K00902 that from the same patient were clustered in one branch next to an Australian isolate NCTC 11637. Nearly all strains from Continental Europe with the definition of hpEurope were assigned into Europe group. All African strains were assigned in Africa group, which located in the farthest end from the centric node, but with one Italian strain be194.

cagA gene

The maximum likelihood tree for the *cagA* gene was shown in *Figure 2*. XZCB-W009, XZCB-K00901, and XZCB-K00902 isolated from the same patient were assigned in one cluster with strain 26695, XZCB-K002 and XZCB-K038 were clustered into another.

The EPIYA motifs in the C-terminal region of CagA protein, encoded by the *cagA* gene, were classified as Western type CagA-ABC (data not shown).

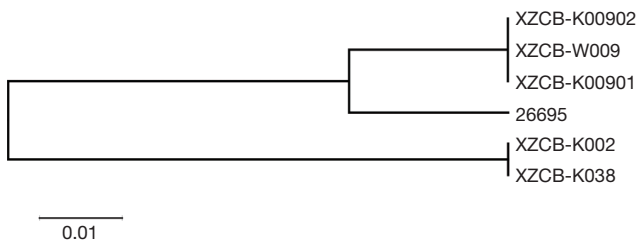

Figure 2 Phylogenetic tree for *cagA* gene of five Tibetan strains and strain 26695.

Electron microscopy of H. pylori

H. pylori SS1, XZCB-W009 and XZCB-K00901, were stained by negative staining and observed under TEM. The bacteria showed 2–6 flagella at one terminal of the body. The flagella of two clinical isolates were richer than SS1, and no significant differences were observed among XZCB-W009 and XZCB-K00901 (*Figure 3*).

Discussion

In this study, we analyzed the genome sequences of five *H. pylori* clinical strains isolated from three patients with gastric diseases in Tibet. Gene loci of SNVs and InDels were detected and identified in the genome of Tibetan strains by comparing to reference genome of *H. pylori* 26695. Based on the viewpoint of molecular genetic evolution, it is interesting finding that in our analysis with MLST, all five Tibetan strains presented more intimate with strains isolated from Australia and New Zealand and were quite different from other prevailing strains in other parts of China. Although Tibetan strains were assigned into the Oceania group and showed hpEurope ancestry, other Chinese strains were grouped as hspEAsia. This is the first study to explore the population structure of *H. pylori* strains epidemic in Tibet of China.

In previous reports, it is shown that according to the MLST analyze, Subpopulations of hspEAsia, hpAsia 2,

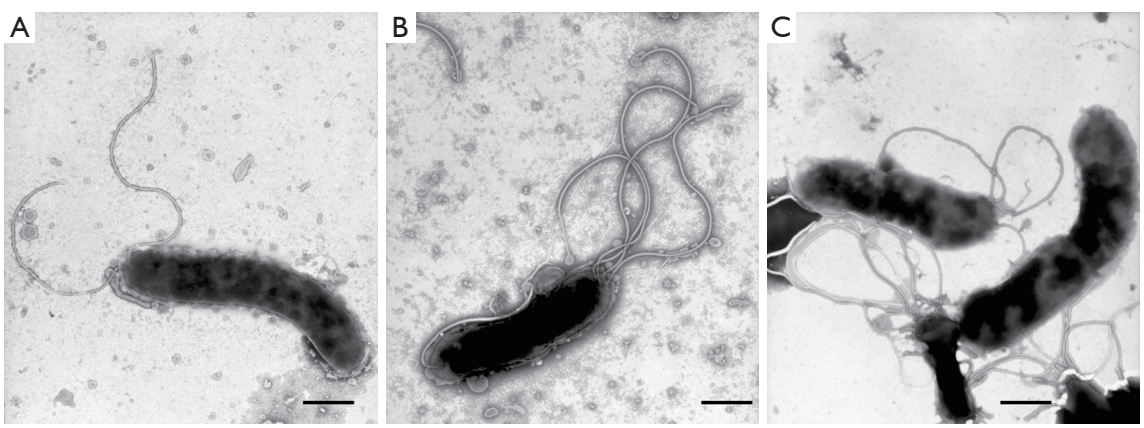

Figure 3 Transmission electron micrographs of the clinical and type strains of *H. pylori*. SS1 (A), XZCB-W009 (B) and XZCB-K00901 (C), harvested from Columbia agar plates at 37 ℃ for about 72 h, negatively stained and observed under JEM-ARM 200F (JEOL) transmission electron microscope, bars, 0.5 μm.

hspMario and hspAmerind were assigned in the Asia group (27,28). Similarly, subpopulations of hpNEAfrica, hspWAfrica, and hspSAfrica were in the Africa group. In our study, the hpEurope population was split into the Oceania and Europe group. It is noteworthy that five *H. pylori* strains from Tibet were all in the Oceania group and defined as hpEurope. However, a previous Tibetan strain XZ274 isolated from a patient with gastric cancer presented a close relationship with strains isolated from southwest China (16,29).

The five Tibetan strains were *cagA*-positive strains. CagA, the product of *the cagA* gene, is delivered into gastric epithelial cells by Type IV Secretion System (TFSS). After tyrosine phosphorylation occurs, CagA specifically binds to SHP-2 phosphatase to activate to phosphatase activity and induce morphological transformation of cells (30). Polymorphism of EPIYA motifs in the C-terminal region of the *cagA* gene leads to pathogenic differences. East Asian CagA is characterized by the tandem arrangement of EPIYA-A, EPIYA-B and EPIYA-D segments in C-terminal region of CagA, whereas Western CagA contains EPIYA-A, EPIYA-B and an indefinite number (one to four) of EPIYA-C segments in tandem (31). The number of EPIYA-C segment is positively correlated with its pathogenicity (32). Accordingly, five strains from Tibet in this study were all defined as Western type CagA-ABC. Notably, East Asian CagA binds to SHP-2 more efficiently than Western type and synthesizes more virulence proteins (33). Moreover, strains of CagA-ABDD show more carcinogenicity than strains of CagA-ABCCC (34).

After isolating *H. pylori* strains from dental plaque of a dental caries patient for the first time in 1989 (35), the evolutionary relationship of *H. pylori* strains in the oral cavity and stomach has drawn much attention, but there is still no definite conclusion. Oral cavity, as an important colonization site of *H. pylori*, plays a significant role in the infection of *H. pylori*. There are differences in morphology and biochemical characteristics among *H. pylori* strains that isolated from different patients, whereas isolates from oral cavity and stomach of the same patient maintain a high-degree consistency (36). Subsequently, the homology of oral and gastric strains is also confirmed by molecular biological methods (37,38). In our phylogenetic analysis, it is confirmed that the gene sequence of three strains that were isolated from the oral cavity and the stomach of the same patient, XZCB-W009, XZCB-K00901, and XZCB-K00902, were almost identical. However, another study showed that from the same patient the agreement between oral and gastric genotypes was only 38.7% (39). In other studies, it is also shown that oral and gastric strains may be completely unrelated (40,41).

In conclusion, five *H. pylori* clinical strains from Tibet were isolated and performed genome sequencing. A huge amount of SNVs/InDels were detected and identified by comparing to *H. pylori* 26695. This study showed that the analyses of MLST improved the definition of *H. pylori* population particularly in Tibet. These Tibet strains were quite different from the strains that were isolated from other parts of China but showed hpEurope ancestry. Moreover, CagA of five Tibet strains showed Western type CagA-ABC, confirming this consequence. However, western CagA, due to less carcinogenic, did not seem to explain the phenomenon of the high prevalence of gastric cancer in the Tibet autonomous region. More Tibetan strains and researches are still needed for further investigation. Also, the relationship between oral and gastric strains from the same patient exhibited homology in molecular evolution.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: All patients were required to provide written informed consent, and all procedures in the study were in complete accordance with the Helsinki Declaration and were reviewed and approved by the Ethics Committee of Hospital of Chengdu Office, People's Government of Tibet Autonomous Region (Approval Number 2015-25).

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