Original Article

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Prevalence of 7 virulence genes of *Legionella* strains isolated from environmental water sources of public facilities and sequence types diversity of *L. pneumopila* strains in Macau

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Summary **In this study, we analyzed 7 virulence genes in 55 *Legionella* species (including 29 *L. pneumophila* and 26 non-*L. pneumophila* strains) which isolated from environmental water sources of the public facilities in Macau by using PCR and real-time PCR. In addition, 29 *Legionella pneumophila* isolates were subjected to genotyping by sequence-based typing scheme and compared with the data reported. The detection rate of *flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA* and *neuA* genes in the *L. pneumophila* were 100.0%, respectively. The *neuA* gene was not detected in the non-*L. pneumophila* strains, but *flaA*, *pilE*, *asd*, *mip*, *mompS*, and *proA* genes could be amplified with a positive rate of 15.4%, 15.4%, 53.8%, 38.5%, 15.4%, and 38.5%, respectively. The results from real-time PCR were generally consistent with that of PCR. Those *L. pneumophila* strains were assigned into 10 sequence types (STs) and ST1 (9/29) was the dominant STs. Four new STs were found to be unique in Macau. The analysis of population structure of *L. pneumophila* strains which isolated from Macau, Guangzhou and Shenzhen indicated that the similar clones were existed and ST1 was the most prevalent STs. However, the distribution of the subtypes isolated from Macau was not the same extensive as those from Guangzhou and Shenzhen. The different detection rates of the 7 virulence genes in different species of *Legionella* might reflect their own potential for environmental adaptability and pathogenesis. And the data analyzed from STs diversity indicated the Macau *L. pneumophila* possessed obvious regional specificity and high genetic diversity.**

Keywords: Sequence-based typing, population structure, phylogenetic relationship

1. Introduction

Legionella species, commonly found in the environment, are the major causative agents of Legionnaire's disease and Pontiac fever. They have been found to not only induce lung infection, but also to cause dysfunction of other organs, such as the heart, kidney and central nervous system (*1*). To date, more than 50 species of *Legionella* have been described (*2*). Among of them, 20 species are recognized as human pathogens. *L. pneumophila* was identified as primary culprit for Legionnaire's disease. In recent years, several studies related to the presence of *Legionella* have been reported in southern Chinese cities, such as Guangzhou, Shenzhen, Jiangmen and Hong Kong (*3,4*). However, no data of *Legionella* from Macau were published. In May of 2010, a case of *Legionella* infection emerged, which was vigorously suspected to be caused by local *Legionella* species since the patient did not previously travel abroad. We investigated and detected the existence of *Legionella* in natural and artificial water environments in Macau in the summer (from May to July) of the same year. A total of 55 isolates of *Legionella* were isolated from air conditioning cooling towers, fountains and

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surface waters in public facilities of Macau (*5*).

In the present study, seven virulence genes (*flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA* and *neuA*) responsible for the expression of adherence, invasion, colonization and cytotoxin production (*6,7*), were detected in all 55 strains isolated from Macau. For comparison, the PCR and the real-time PCR were applied to detect the genes simultaneously. In addition, sequence-based typing (SBT), a powerful epidemiological method recognized by the European Working Group for Legionella Infections (EWGLI) as a "gold standard" tool, was used for the *L. pneumophila* strains. The population structure and phylogenetic relationship of *L. pneumophila* strains isolated from Macau were analyzed and compared to those from near cities such as Guangzhou and Shenzhen. This investigation will help us to learn more about the characteristics of the Macau *Legionella* isolates, provide the possibility to trace and control for a large area of epidemic and appropriate precaution strategy against *Legionella* infection.

2. Materials and Methods

2.1. *Legionella strains*

A total of 55 *Legionella* strains were collected from 43 water samples including air conditioning cooling towers (27 samples), fountains (13 samples) and surface waters (3 samples) in public sites of Macau. *Legionella* species were identified by serological agglutination with Legionella Latex Agglutination Kit (PRO-LAB, Weston, USA) based on manufacturer instructions and fatty acid analysis was performed with the Sherlock microbial identification system (software version 6.0, MIDI, USA; Microbial ID, Inc., Newark, Del).

2.2. *Preparation of DNA*

The strains were cultured with buffered charcoal yeast extract (BCYE) ager plates at 37°C in 5% CO_2. A single colony of *Legionella* was picked from the plate and resuspended into 100 μL sterilized ultrapure water. DNA was then extracted by one thaw-freeze cycle (99°C for 10 min and 4°C for 5 min). After briefly centrifuged, the supernatant was measured with a spectrophotometry at 260 nm in triplicates using A260/A280 ratio (NanoDropTM 1000, Thermo Scientific) then used as the DNA template for PCR and real-time PCR.

2.3. *Detection of pathogenic genes by PCR and real-time PCR*

The PCR primers were based on EWGLI recommended (*8,9*). PCR conditions were the same as described by Gaia *et al*. (*8*) with minor modification. PCR was performed in a mixture (50 μL) consisting of 25 μL of 2× SuperStar PCR mix (GenStar Biosolution, Beijing, China), 5 pmol each primers, 100 ng of the template. Sterile distilled water was added to make 50 μL. The products were detected by electrophoresis and purified using the DNA Gel Extraction Kit (Axygen USA).

To establish a more sensitive, higher speciality and easier operation detecting method, a real-time PCR was applied at the present study. The primers used in real-time PCR were designed based on sequences from GenBank accession numbers X83232 (*flaA*), AF048690 (*pilE*), AF034213 (*asd*), AJ496269 (*mip*), AF078136 (*mompS*), M32884 (*proA*) and AJ007311 (*neuA*). The primers sequences were list in Table S1. For amplifications, 50 ng template was mixed with 12.5 μL 2× SYBR Premix EX Taq™ II (Perfect Real Time, Takara, Japan), and 5 pmol each of the forward and the reverse primers in a final volume of 25 μL. The reaction conditions were 5 s at 95°C and 30 s at 60°C for 40 cycles. Sterilized water was used as a template for the blank control for both PCR reactions.

2.4. *Sequence-based typing*

The purified PCR products were sequenced by Beijing Genomics Institute (Beijing, China). SBT using loci *flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA* and *neuA* was performed according to the EWGLI scheme (*8,9*). Genotype analysis was based on the standard SBT method given by the EWGLI with these 7 genes. The nucleotide sequences obtained were confirmed by the SBT database available on the EWGLI website (*http://www.ewgli.org/*), and the sequences were compared with those in the SBT database from the website (*http://www.hpabioinformatics.org.uk/legionella/legionella_sbt/php/sbt_homepage.php*).

2.5. *Population structure and phylogenetic analysis*

The minimum spanning trees (MST) were conducted by the BioNumerics software (version 7.1; Applied Maths, Kortrijk, Belgium). In MST, the ST that possesses the most number of single-locus variants is defined as the founder ST. The clusters of relative STs that originate from a common ancestor are considered as the clone groups or complexes. The single genotype that does not correspond to any other clone groups is classified as singleton. The sequence types are represented by the circles. The size of circle indicates the number of the particular strains. The relationship of the different circles is present with the connecting lines.

The concatenated sequence in phylogenetic analysis was prepared with the seven loci of the initial SBT scheme according to their locations on the chromosome by BioEdit (*http://www.mbio.ncsu.edu/BioEdit/bioedit.html*). Based on the concatenated sequence, the evolutionary relationship between STs was conducted using the neighbour-joining method with Tamura

Table 1. Detection rate of seven genes in *Legionella* strains isolated from Macao

Species	No. of strains	No. of positive strains													
		flaA		*pilE*		*asd*		*mip*		*mompS*		*proA*		*neuA*	
		P[a]	R[b]	P	R	P	R	P	R	P	R	P	R	P	R
L. pneumophila	29	29	27	29	29	29	29	29	28	29	29	29	29	29	29
L. adelaidensis	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L. rubrilucens	3	1	0	0	0		0	0	0	1	1	0	0	0	0
fluoribacter-gormanii	3	0	0	2	1	3	0	0	0	0	0	0	0	0	0
L. shakespearei	5	0	0	0	2	5	4	5	3	1	3	5	4	0	3
L. feeleii	6	0	0	0	0	0	0	0	0	1	4	0	0	0	2
L. wadsworthii	3	2	2	0	0	3	1	0	0	0	0	1	3	0	0
L.quateirensis	5	1	0	2	2	3	0	5	3	1	4	5	3	0	0
		Detection rate of seven genes with three different methods (%)													
L. pneumophila	29	100	93.1	100	100	100	100	100	96.5	100	100	100	100	100	100
No-*L. pneumophila*	26	15.4	7.69	15.4	19.2	53.8	19.2	38.5	23.1	15.4	46.2	42.3	38.5	0	19.2

[a]PCR; [b]Real-time PCR.

Table 2. Sequences-Based Typing of *L. pneumophila* strains from Macao

SBT type	*flaA*	*pilE*	*asd*	*mip*	*mompS*	*proA*	*neuA*	Serotype (no.)	No. of isolate
1	1	4	3	1	1	1	1	Lp1(5)LP14(4)	9
160	11	14	16	16	15	13	9	Lp1(1)Lp14(1)	2
566	16	4	3	1	1	1	1	Lp1(2)Lp14(1)	3
752	22	4	3	1	1	1	1	Lp1	1
1119	2	10	14	10	21	4	3	Lp1	2
1417	8	6	34	9	2	8	209	Lp14	4
01[a]	11	14	16	25	15	13	206	Lp14	1
02[a]	11	4	16	16	15	13	9	Lp1	1
03[a]	1	4	3	5	11	1	15	Lp14	4
04[a]	11	4	16	12	15	13	9	Lp1(1)Lp14(1)	2

[a] New ST type assigned by SBT database of EWGIL.

3-parameter model by MEGA v5.05.

3. Results

3.1. *Legionella isolates*

A total of 55 isolates of *Legionella* were isolated from air conditioning cooling towers (96.8%), fountains (1.8%) and surface waters (1.8%). Among them, *L. pneumophila* accounted for 29 isolates, approximately 52.7% of all isolates, whereas *Legionalla* species other than *L. pneumophila* accounted for 47.3% of the total. Among the 29 *L. pneumophila* species, the serotype 1 strains accounted for 44.8%, whereas serotype 14 accounted for 55.2%. *L. feeleii* was the dominant species (23.1%) among 26 non-*L. pneumophila* species, followed by *L. shakespearei* and *L. quateirensis*, which severally accounted for 19.2%. The above indicated that *Legionella* species were widely distributed in the public environment of Macau and *L. pneumophila* occupied the major proportion.

3.2. *Different prevalence of pathogenic genes in Legionella species*

In 29 *L. pneumophila* strains, *flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA* and *neuA* genes were 100% detected (Table 1). In 26 non-*L. pneumophila*, other genes included *flaA*, *pilE*, *asd*, *mip*, *mompS*, and *proA* which tested as 15.4%, 15.4%, 53.8%, 38.5%, 15.4% and 38.5%. However, the *neuA* gene failed to amplify. The positive results of the three genes *asd*, *mip* and *proA* in non-*L. pneumophila* focused on the *L. quateirensis* and *L. shakespearei* strains.

For *L. pneumophila* strains, the results of real-time PCR were almost same as that of PCR, but the positive rate of *flaA* and *mip* genes were 93.1% and 96.5% (Table 1). The positive results for seven genes of non-*L. pneumophila* from real-time PCR exhibited some differences with that from PCR. Genes of *flaA*, *asd*, *mip* and *proA* showed a little lower sensitive while *pilE*, *mompS* and *neuA* genes were with more sensitive. In summary, the detection rates of seven genes were high in *L. pneumophila* strains and relatively low in non-*L. pneumophila* strains.

3.3. *Sequence-based typing*

The 29 environmental *L. pneumophila* strains could be divided into 10 STs (Table 2), including 3 singletons, in which one was ST752 and the other two were new STs (ST01 and 02). According to the EWGLI SBT

database submitted in March 2014, there were four new STs unique to Macao. The ST with the largest number of isolates was ST1 (9/29, 31.0%), followed by ST1417 and ST03 (4/29, 13.8%), ST566 (3/29, 10.3%), then ST160, ST1119 and ST04 (2/29, 6.9%). There were 36 alleles were identified across the 7 loci. At the individual loci level, *mip* gene provided the most alleles. Seven alleles were obtained from *mip* gene, whereas six alleles were obtained using *flaA* and *neuA* genes, five from the *mompS* gene, and four from *pilE*, *asd* and *proA* genes. The results revealed that ST1 was the main ST-type and *mip* gene showed greatest genetic diversity in Macau.

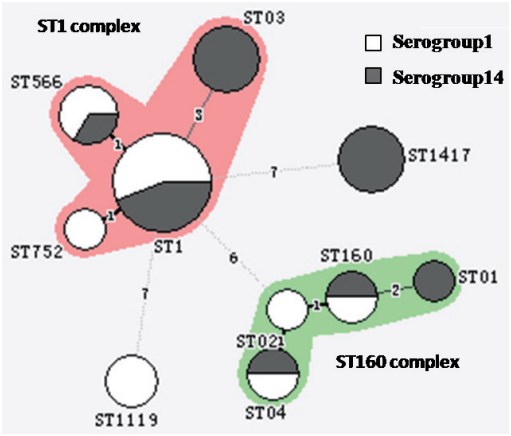

Figure 1. MST analysis of 29 *L. pneumophila* strains isolated in Macau. STs are represented by circles. The size of each circle indicates the number of isolates within this particular type; the STs are shown beside the circles and the number of isolates in each ST is shown in the circles. The branch distance is shown on the connecting line. This links STs or sets of STs with a branch distance less than or equal to 3 within a single ST complex.

3.4. *Population structure and Phylogenetic analysis*

All 10 STs identified from Macau were analysed for their population structure with the minimum spanning trees (Figure 1). Among them, 8 STs formed two clone complexes, and the other 2 different STs were determined to be singletons with four or more gene variants. The ST1 complex was a bigger clone group, including four STs groups containing 17 isolates. ST1 was suggested to be a primary founder, since there were 9 ST1 (9/17, 52.9%), the highest number of isolates in the complex. The other three STs in the ST1 complex were either single-locus variant or double-locus variants to ST1. In the other clone group ST160 complex, there were 4 STs with 6 isolates. Three (ST01, 02 and 04) of four new STs unique to Macao were found in this complex, while ST03 belonged to the ST1 complex.

The 29 strains in the study with the addition of 116 isolates from Guangzhou (*10*), and 52 isolated from Shenzhen (*11*) were put together and analysed again with minimum spanning tree by the BioNumerics software (Figure 2). They were identified as 47 STs, of which, 43 STs were grouped into eight clonal complexes. The other 4 STs (ST59, 114, 238, 1054) were singletons. Only ST1, ST 160 and ST752 were found in the three areas. The isolates from Macau were distributed mainly in two large clone complexes, I and II. Only one Macau STs could be found in the complexes III and VIII.

Clone complex I, the largest clone group in these three cities, contained the ST1 complex from Macau. There were four main STs, ST150, ST154, ST159 and ST160 in clone complex II, in which most of STs were single-locus or double-locus variants to ST154

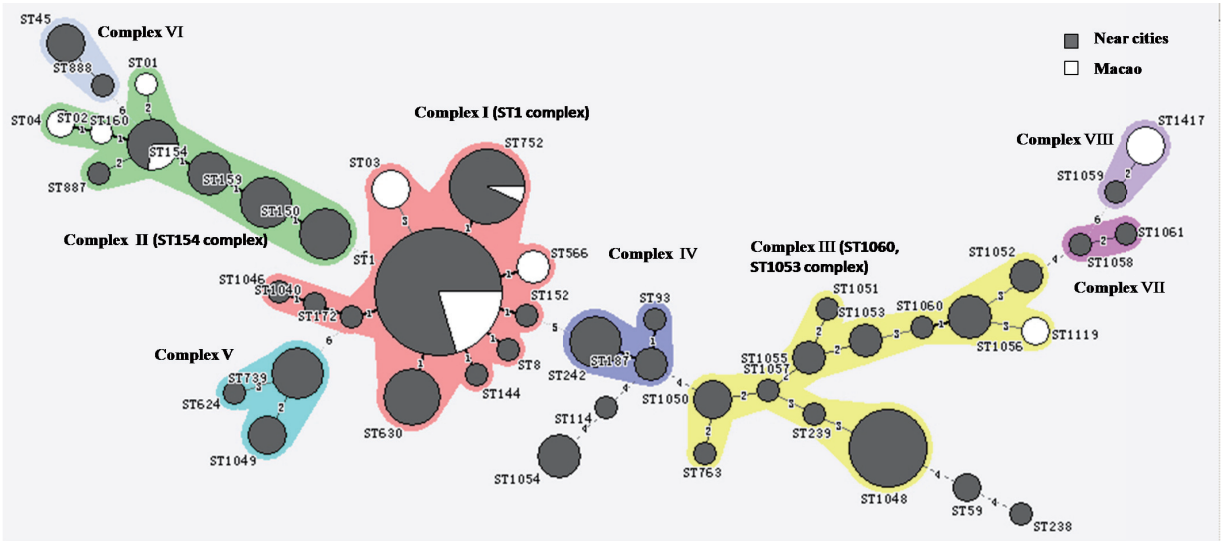

Figure 2. MST analysis of 197 *L. pneumophila* strains isolated from Macau, Guangzhou and Shenzhen. STs are represented by circles. The size of each circle indicates the number of isolates within this particular type; the STs are shown beside the circles. The heavy solid black lines connect two types within a single variant locus, the light solid black lines connect double-locus variants, the light solid gray lines connect triple-locus variants, the gray dashed lines connect quadruple-locus variants, and the dotted lines connect variants over quadruple-locus. The branch distance is shown on the connecting line. The colors of the halo surrounding the STs denote types that belong to the same clonal group.

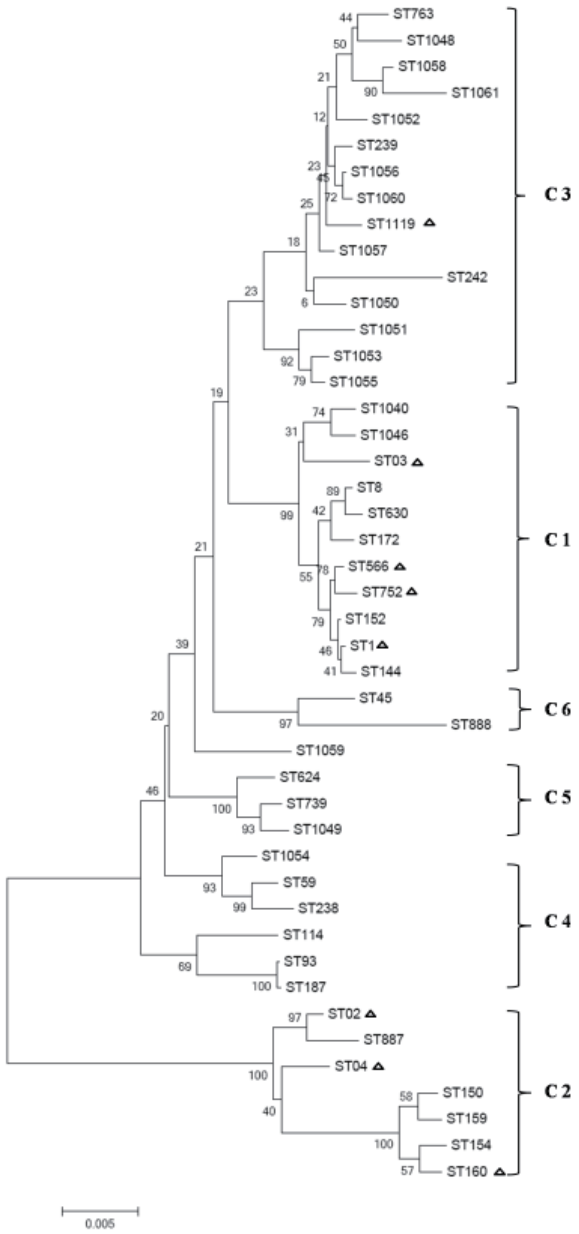

Figure 3. Phylogenetic analysis of the *L. pneumophila* STs isolated from Macau, Guangzhou and Shenzhen. The tree was constructed with MEGA v5.05. STs of strains isolated from Macao are labeled by black triangles. The scale bar indicates genetic distances between sequences. The percentages of replicate trees in which the associated STs clustered together in the bootstrap test are shown next to the branches. The evolutionary distances are in the units of the number of base substitutions per site.

or ST160. Clone complex III was another large group, which composed of the most STs with double-locus or triple-locus variants each other. The STs in the other four small clone complexes, IV to VII were from Shenzhen and Guangzhou, but not Macau.

STs phylogenetic analysis was conducted using a neighbor-joining method with the Tamura 3-parameter model based on concatenated sequences of SBT alleles. Because ST01 and ST1417 were obtained the *neuA* allele with new primers, the results of their phylogenetic analysis were unfaithful. The other STs were divided into six groups (C1-C6) by the neighbor-joining analysis (Figure 3). These groups generally had a one-to-one correspondence with the six clonal complexes (complexes I to VI) determined by BioNumerics software. It was unexpected that the single C3 group identified by MEGA v5.05 contained both clonal complexes (III and VII). The distribution of Macau STs wasn't as wide as that of STs from near cities Guangzhou and Shenzhen, and ST1 was the preponderant STs among these three cities.

4. Discussion

We have previously studied the distribution and species of environmental *Legionella* isolates from Macau in detail (*5*). In this work, our main objective has been to analyze the prevalence of seven virulence genes of this species and reveal the STs distribution of *L. pneumophila* isolates from Macau. Furthermore, the *L. pneumophila* isolates from Macau were compared with the isolates from Guangzhou and Shenzhen. Our findings revealed the high prevalence of these seven virulence genes in *L. pneumophila* strains and low prevalence in non-*L. pneumophila*. Additionally, our study also indicated that STs had several unique allelic profiles and ST1 is the predominant ST-type in Macau.

The seven genes detected in this study are closely related to bacterial signal transduction, virulence, and adaptive capacity (*6,12-14*). The different detection rates of these genes revealed the relationships between each virulence gene or combinations of these genes and the various species of *Legionella* in Macau. The high prevalence of these genes in *L. pneumophila* strains and low prevalence in non-*L. pneumophila* strains might reflect their own ability to adapt to the external environment and their strong pathogenicity to human beings. Some reports have shown that non-*L. pneumophila* could cause a variety of organ dysfunction (*15*). However, studies focused on detection of virulence genes associated with non-*L. pneumophila* strains are rarely found. This study represents the first detection for these seven virulence genes from both *L. pneumophila* and non-*L. pneumophila* strains which might provide a reliable basis to evaluate the pathogenic potential of the strains and their adaptability to the local environment.

In this study, the real-time PCR was also applied for its high level of efficiency and ability for specialization, as well as for its ability to multiply amplify the target genes and its ease of operation. The results from real-time PCR were generally consistent with that of PCR. The data suggested that real-time PCR, instead of conventional PCR, should be used to detect these seven genes, especially for large-scale investigation. If the PCR products needed to be collected for other purposes, or used to perform a test to compare and verify the relevant multi-alleles, both tests could be

used together. Since the primers designed for real-time PCR were not appropriate for all *L. pneumophila* and non-*L. pneumophila* strains, a future study is needed to further investigate this issue.

These 29 *L. pneumophila* isolates were classified with the method recommended by EWGLI and four new STs were found unique to Macau. Within the 10 Macao STs, ST1 is the predominant ST-type (9/29, 31.0%). The result was similar to the report in Japan where STI consisted of 29% of environmental isolates (*16*). In a recent study conducted in the United States, ST1 accounted for 25% and 49% of the number of the sporadic and environmental isolates, respectively (*17*). In one study conducted in England and Wales, ST1 was the most frequent STs, accounting for 35% of the number of *L. pneumophila* environmental isolates (*18*). From the *L. pneumophila* serogroup 1 isolates from potable systems, cooling towers, and hot springs in China, ST1 were reported to be about 14.3%, to 53.1% and 92.3%, respectively (*19*). For *L. pneumophila* strains, ST1 (1, 4, 3, 1, 1, 1) is predominant in environmental samples, widely distributed around world (*18,20,21*). The Macau SBT analysis of *L. pneumophila* strains isolated from the public sites revealed the same ST-type characterization.

In the study, the loci *mip* and *flaA* offered more alleles in Macau's samples. The number of each allele presented by EWGLI from all over the world showed that the *mompS* locus provided the maximum number of alleles, *neuA-Ah* provided the next most, and *mip*, *asd*, *proA* and *pilE*, *flaA* provided the minimum. One report in South Korea showed that the *mompS* locus had the most alleles (*2*). Similarly, another study conducted in Canada obtained the same result that locus *mompS* provided the most alleles (*22*). However, one study on SBT of *L. pneumophila* strains in mainland China indicated that *mip* and *flaA* individually provided the most alleles in the isolates from cooling water and hot spring water (*19*). This might be attributed to the geographical correlation which could lead to the emergence of these results. Additionally, it might also reflect that the homology that exists between strains from Macau and mainland China.

Meanwhile, in order to understand the population structure and phylogeny of the *L. pneumophlia* strains isolated from Macau, the minimum spanning tree and the neighbor-joining method were respectively applied to compare Macau STs to those identified from nearby cities of Guangzhou and Shenzhen. All STs from these three cities were divided into eight clone complexes in the minimum spanning tree, and six groups could be seen by the neighbor-joining method. Consistent results from these two similar methods suggested that the phylogenetic analysis could be selected either individually or simultaneously. Among eight complexes generated in the minimum spanning tree, only four were related to Macau STs. The subtype distribution of *L. pneumophlia* strains isolated from Macau was not as extensive as that from other cities (*20,22,23*). However, in complex I, ST1 from Macau was the major STs involved in, which reflects the concentration of local types. This supports that ST1 is the most common hereditary character of *L. pneumophlia* strains and extensively distributed all over the world. The majority of isolates from Macau kept also represented this distrubution. In this study, four new STs were found for the first time. It is convinced of that more new STs will be reported in further investigation. After a detailed study using phylogenetic analysis and population structure, the high diversity and specificity of *L. pneumophlia* strains isolated from Macau were observed. Since ST154 has been proven to be relevant to *Legionnella* epidemiology (*1,2*), the closely connected ST04, ST02 and ST160, should bring significant attention and research interest.

To summarize, this study enables, for the first time, the ability to realize the prevalence of seven virulence genes of *Legionella* in Macau, to characterize the *L. pneumophila* environment isolates with SBT methodology, and to create a database of Macau's *L. pneumophila* profiles for use in epidemiological surveillance efforts. The findings of this study also contribute to the EWGLI-SBT database and to the knowledge of *L. pneumophila* diversity in southern China. Further studies are needed to reveal the relationships between each pathogenic gene or combinations of these genes and the pathogenicity of *Legionella*, and to analyze the correlation between environmental and clinical strains of *Legionella*.

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Supplemental data

Table S1. Sequence of primers designed for detecting virulence genes used in real-time PCR

Gene	Primers (5'-3')	Product size (bp)
flaA	F: GATGCTACGTCTGCCTAT R: CCTGCGGTTCCACCTATT	119
pilE	F: CGATGCTCATGCCACATT R: CCGTTCGGAGTTGTTTGC	120
asd	F: AAGCGGTTCATCTGGAGT R: TGCTGTGGGATAACTTGC	119
mip	F: AAATGCCATCGTTCCTG R: AGAAGCTGCGAAATCAGT	164
mompS	F: TGCCATCGTTCCTGAGTT R: GACCAGAAGCTGCGAAAT	164
proA	F: GGTGCTGTAGTTTCAACG R: GTGGCATTCTTACTGTGC	143
neuA	F: TGCCTTGCAGTCGTCTTG R: TCCGTGGCTAAATCTTCC	123

Primer designed with the sequences downloaded from EWGLI (http://www.ewgli.org/) as a template. *F* forward, *R* reverse.