

SHORT REPORT

Combined use of real-time PCR and nested sequence-based typing in survey of human *Legionella* infection

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SUMMARY

Legionnaires' disease (LD) is a globally distributed systemic infectious disease. The burden of LD in many regions is still unclear, especially in Asian countries including China. A survey of *Legionella* infection using real-time PCR and nested sequence-based typing (SBT) was performed in two hospitals in Shanghai, China. A total of 265 bronchoalveolar lavage fluid (BALF) specimens were collected from hospital A between January 2012 and December 2013, and 359 sputum specimens were collected from hospital B throughout 2012. A total of 71 specimens were positive for *Legionella* according to real-time PCR focusing on the 5S rRNA gene. Seventy of these specimens were identified as *Legionella pneumophila* as a result of real-time PCR amplification of the *dotA* gene. Results of nested SBT revealed high genetic polymorphism in these *L. pneumophila* and ST1 was the predominant sequence type. These data revealed that the burden of LD in China is much greater than that recognized previously, and real-time PCR may be a suitable monitoring technology for LD in large sample surveys in regions lacking the economic and technical resources to perform other methods, such as urinary antigen tests and culture methods.

Key words: Bacterial infections, clinical microbiology, infectious disease, *Legionella*, Legionnaires' disease.

Legionnaires' disease (LD) is a form of atypical pneumonia caused by Gram-negative bacteria of the genus *Legionella* [1]. LD is reported less commonly in Asian countries, especially in China. In the past 10 years,

cases of infection by *Legionella* have been reported in China only sporadically or in small outbreaks [2–5]. In China, most patients use antibiotics prior to hospital admission, and it is difficult to obtain strains from respiratory specimens. In a previous report, we described four cases of *Legionella pneumophila* infections associated with liver cirrhosis; of these cases three were identified by real-time polymerase chain reaction (real-time PCR) [4].

Isolation of *Legionella* is considered the 'gold standard' for the diagnosis, but culture-confirmed

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† There was an error of omission in the title that has now been corrected and a notice has been published providing details.

Table 1. Details and positive rate of respiratory specimens tested in the study

Hospital	Period of sample collection	Sample type	No. of samples	No. of positive samples	Positive rate
Hospital A	Jan. 2012–Oct. 2013	BAL	265	41	15.47%
Hospital B	Jan. 2012–Dec. 2012	Sputum	359	30	8.36%

BAL, Bronchoalveolar lavage.

diagnosis of *Legionella* infections is often hampered by early antibiotic treatment and demanding culture requirements. The urinary antigen (UAG) test is commonly used to diagnose LD; however, the present UAG test reagents could detect only *L. pneumophila* serogroup 1 (Lp1) with non-Lp1 LD being missed. Furthermore, because of its high cost, the UAG test is unlikely to be widely used in economically underdeveloped regions. The benefit of performing PCR methods on respiratory samples is now clearly established, especially for diagnosing other species and serogroups than Lp1 [6–8]. In a study conducted by Murdoch *et al.* in New Zealand, a >fourfold increase in the number of detected cases of LD by real-time PCR compared to culture was obtained [8]. In the present study, we undertook a retrospective survey to detect *Legionella* in patients with pneumonia or other lower respiratory tract infections (LRTIs). Nested sequence-based typing (SBT) was used for analyses of the population structure of real-time PCR-positive specimens.

A real-time PCR-based survey was performed in two hospitals in Shanghai, China. Real-time PCR focusing on the 5S rRNA gene, which has a specificity of 100% and a sensitivity of 10 copies of genomic DNA, as described previously, was undertaken [4, 9, 10]. Real-time reactions were conducted in a quantitative PCR instrument (Stratagene, USA) and conditions were as follows: an initial denaturing step of 95°C for 2 min, followed by 40 cycles of 95°C for 10 s and 60°C for 20 s. During the study period, patients were sampled who had symptoms of pneumonia or other LRTIs (cough, expectoration, moist rales on lung, and one of the following: fever, neutrophilic infiltration, or changes by chest radiography) and where *Streptococcus pneumoniae* and *Haemophilus influenzae* had been excluded. A total of 265 bronchoalveolar lavage fluid (BALF) specimens were collected from hospital A between January 2012 and December 2013, and 359 sputum specimens were collected from hospital B throughout 2012. All specimens were analysed by culture method using buffered charcoal-yeast extract agar with α -ketoglutarate (BCYE; Oxoid, UK)

and no *Legionella* isolates were obtained from any specimen. These specimens were collected in our laboratory and tested for *Legionella* by real-time PCR. The overall positive rate among 624 cases was 11.4%. A total of 71 specimens were positive for *Legionella* according to real-time PCR: 41 (15.5%) BALF specimens from hospital A and 30 (8.4%) sputum specimens from hospital B (Table 1). Real-time PCR focusing on the *dotA* gene (which is restricted in *L. pneumophila*) was performed to test the 71 specimens [11]. Seventy were identified as *L. pneumophila* as a result of real-time PCR amplification of the *dotA* gene.

The 71 cases of *Legionella* infection were aged 23–76 (median 58.4) years and 48 (67.6%) were male. All 71 cases were community acquired as the symptoms were observed before hospital admission. Upon hospital admission, six (8.5%) of these cases were diagnosed with pneumonia, 43 (60.6%) had lung shadows, 17 (23.9%) had fever with cough, three (4.2%) had pleural effusion, and two (2.8%) had a lung abscess. All 71 patients had changes consistent with pneumonia according to chest radiography after hospital admission. In hospital A, positive rates were slightly higher during the first and fourth quarters than in the second and third quarters, especially in 2012. In hospital B, the number of positive cases and positive rates were higher in the fourth quarter than in the other quarters (Fig. 1). There is trend of seasonal variation of positive rates and case numbers, in particular the autumn peak is consistent with the pattern found in other regions [12]; however, a survey with a larger sample size should be performed to obtain a more accurate conclusion.

All 71 real-time PCR-positive specimens were tested by nested SBT using a protocol described previously [13]. A full 7-allele profile was obtained for 44/71 (62.0%) specimens, a further 13/71 (18.3%) specimens gave 5- or 6-allele profiles [usually sufficient to identify the strain as belonging to one or two sequence types (STs)], 4/71 (5.6%) gave 3- or 4-allele profiles (usually sufficient to differentiate one profile from another), and 9/71 (12.7%) gave 1- or 2-allele profiles

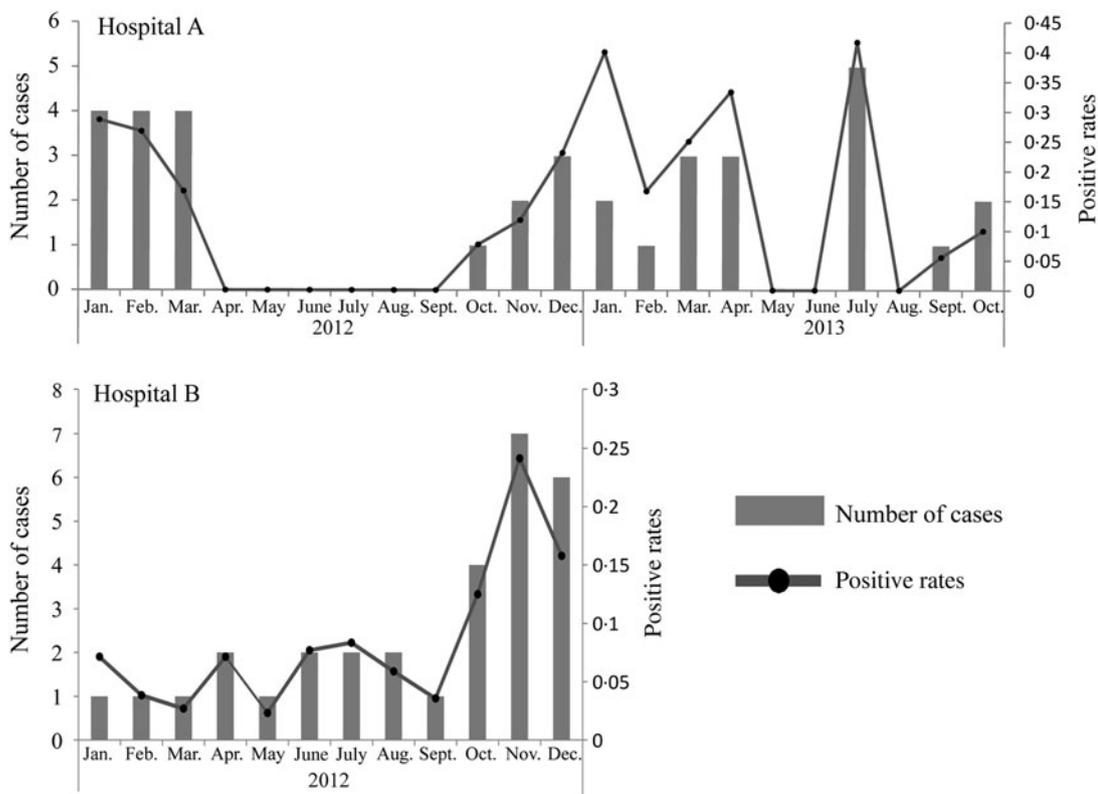


Fig. 1. Number of positive cases and positive rates of cases of *Legionella* infection detected by real-time PCR in two hospitals, China.

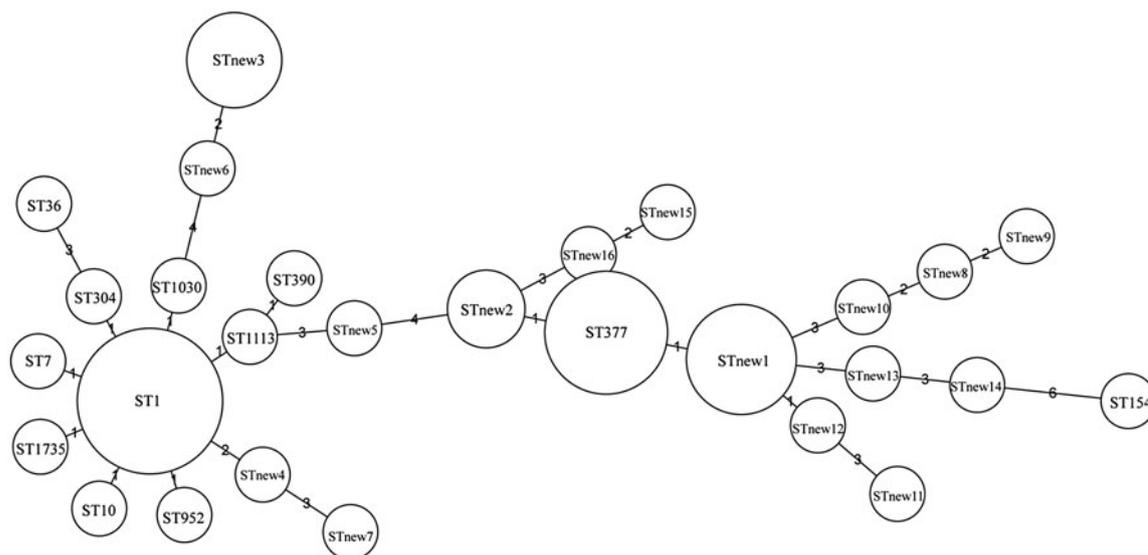


Fig. 2. Minimum spanning-tree analyses of 44 specimens with a full 7-allele profile obtained based on sequence-based typing. Sequence types (STs) are shown as circles. The size of each circle indicates the number of isolates within this particular type. STs are shown in circles; blank circles represent new STs.

(sufficient to distinguish strains). Overall, 70/71 (98.6%) gave at least one allele. Of 41 BALF specimens, 80.5% ($n = 33$) gave a full profile. Only 36.7% (11/30) sputum specimens gave a full profile, and

40.0% (12/30) of sputum specimens gave 5- or 6-allele profiles (Supplementary Table S1).

Of 44 specimens in which the full 7-allele profile was obtained, 28 STs were identified with a

Simpson diversity index of 0.9567, suggesting high genetic polymorphism [14]. A minimum spanning tree based on 28 STs was structured using BioNumerics v. 7.1 software (www.applied-maths.com) (Fig. 2). Of the 28 STs, 12 could be assigned a ST number and the other 16 STs were identified for the first time using the EWGLI SBT database (www.ewgli.org). ST1, ST154 and ST377 were also detected in our previous environmental surveys in China [15, 16]. Of these three STs, ST1 was the predominant ST in strains isolated from all types of water systems [16]; the ST377 strain was detected in hot springs [15]; ST154 was detected in cooling towers and hot springs [16]. This data is consistent with the widely recognized role that cooling towers and hot springs play in the causation of LD. ST1 is also the predominant ST of clinical and environmental isolates worldwide [16–18]. Seven STs (ST7, ST10, ST304, ST952, ST1030, ST1113, ST1735) in this study were single-locus variants (SLVs) of ST1. Two specimens that gave 6-allele profiles were in accordance with that of ST1. ST377 (6-10-15-28-9-14-1) was the second most predominant ST in this study, and was contained in five specimens.

The current system used to monitor *Legionella* in China is focused on environmental water samples rather than clinical samples [19, 20], and a systematic survey has not been reported. The major finding from this study is that the burden of LD in China is much greater than recognized previously. However, data regarding the UAG test is lacking and the prevalence of LD in this study is probably underestimated as several studies have shown that the UAG test can be more sensitive than PCR in respiratory samples for diagnosing LD [21]. Combining real-time PCR and the UAG test may have better application for diagnosing LD. A systemic survey synchronous with UAGs, PCR and culture should be conducted in China in the future. Furthermore, using nested SBT allows extending the results of population structure to uncultivable clinical *Legionella* strains.

SUPPLEMENTARY MATERIAL

For supplementary material accompanying this paper visit <http://dx.doi.org/10.1017/S0950268815003301>.

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DECLARATION OF INTEREST

None.

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