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Two-Step Scheme for Rapid Identification and Differentiation of *Legionella pneumophila* and Non-*Legionella pneumophila* Species▽

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A rapid two-step scheme based on PCR amplification and enzymatic digestion analysis of a 226-bp fragment of the 16S rRNA gene was developed to identify the *Legionella* genus by PCR amplification and to differentiate the *Legionella pneumophila* and non-*Legionella pneumophila* species by enzymatic digestion analysis. Among 42 ATCC strains (16 strains of *L. pneumophila* and 26 strains of non-*L. pneumophila*) and 200 *Legionella* isolates from environmental water samples, including pools, rivers, lakes, and cooling towers in Guangdong province, 99.59% of *L. pneumophila* and non-*L. pneumophila* strains were correctly identified and differentiated by this scheme. The procedure of this two-step identification and differentiation scheme is simple and takes only about 4 h. These results suggest that this two-step scheme provides a simple and convenient method for the rapid identification and differentiation of *L. pneumophila* and non-*L. pneumophila* species.

Legionella species, which are fastidious and ubiquitous worldwide in natural water environment such as rivers, lakes, and artificial water systems, are the causative agent of Legionnaire's disease (7, 23). *L. pneumophila* is the most common pathogenic species within the genus *Legionella* and is the main cause of Legionnaire's disease, which appears as a mild respiratory illness, an acute life-threatening pneumonia, or Pontiac fever (25). Many *Legionella* species have been recognized as human illness agents (2). In addition to *L. pneumophila*, 20 *Legionella* species have been documented as human pathogens on the basis of their isolation from clinical material (4), but they occur at very low frequencies (12). Non-*L. pneumophila* species also have been reported to be infectious (9). The majority of the confirmed infections involving non-*L. pneumophila* were from immunosuppressed patients (4).

Clinical manifestations caused by *Legionella* infection usually are indistinguishable from the pneumonia of other bacterial etiologies. Since the symptoms are atypical, it is difficult to clinically identify the actual causative agent (4). Therefore, the identification of *Legionella* species and the differentiation of *L. pneumophila* and non-*L. pneumophila* species have been of increasing importance (2).

Current methods for the detection of *Legionella* species are based on culture techniques, which take at least 3 to 10 days. The long turnaround time (TAT) limits these methods for their clinical utility. Additional problems with culture detection include low sensitivity, the requirement of special media, adequate specimen processing, the need for technical experts, and microbial contamination inhibiting *Legionella* growth. Contamination by some other microorganism that is viable but nonculturable can appear after the 3 to 10 days of culture for *Legionella* because of the fastidious nature of the these microorganism and the requirement of prolonged incubation periods for their growth. These problems make *Legionella* isolation and identification challenging (3, 6, 15).

Methodologies exploited for the identification of *Legionella* isolates include direct fluorescent antibody (DFA), urine antigen detection (5, 11), and sequence-based genotypic classification schemes such as PCR and real-time PCR (2, 10, 19, 21). Urine antigen detection is the most widely used and is considered to be specific for *L. pneumophila* serogroup 1, but it misses about 40% of legionellosis cases (5, 11). DFA has a low sensitivity for the diagnosis of respiratory samples. These two methods cannot detect non-*L. pneumophila* spp. Although *L. pneumophila* is the most frequent cause of legionellosis, non-*L. pneumophila* species also may cause serious or fatal disease (16, 24). Non-*L. pneumophila* species in respiratory specimens are not detectable by the *L. pneumophila* direct fluorescent antigen test. Similarly, the *Legionella* urinary antigen test will be negative for urine specimens from patients with infections caused by these bacteria, and fluorescent antibody stains are not commercially available for the identification of non-*L. pneumophila* isolates (22). Therefore, the rapid identification and differentiation of *L. pneumophila* and non-*L. pneumophila* is of critical importance for the diagnosis.

Several sequence-based genotypic classification schemes have been reported to identify and differentiate *Legionella* species in laboratories. A multiplex PCR assay was used to detect and differentiate *L. pneumophila* and non-*L. pneumophila* species by targeting a 386-bp fragment of the 16S rRNA gene and the macrophage infectivity potentiator (*mip*) gene fragment. This method had low specificity because the *mip* special primers for *L. pneumophila* species were not specific (19).

Real-time PCR has some benefits compared to routine diagnosis, as it can minimize the manual time for the PCR and make the use of post-PCR analysis convenient. These diagnostic PCR assays targeted specific regions, including the 16S rRNA gene (10, 18, 23), the 23S-5S spacer region (8), the 5S

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rRNA gene (13, 14), and the *mip* gene (3, 17). They can detect only the genus *Legionella* and cannot differentiate *L. pneumophila* from non-*L. pneumophila* species.

The use of the sequence of the *mip* gene was reported to be able to accurately discriminate among 39 *Legionella* species, because the *mip* gene was specific to most *Legionella* members and the subsequent interspecies sequence variation was sufficient to discriminate clearly between species, and it enabled the species-specific identification of most *Legionella* species implicated in human disease (17). A sequence-based classification scheme based on the smaller 5S rRNA gene (104 bp) and partial 16S rRNA gene sequencing was less discriminatory than that's with the *mip* gene (14, 17). However, both methods were complex and time-consuming.

The use of partial 16S rRNA gene sequencing for the identification of *L. pneumophila* and non-*L. pneumophila* species was another reported method to differentiate the *Legionella* species. It was based on gene sequencing and alignment. This method also was a time-consuming method, and its specificity has been questioned (22).

For all of the reasons mentioned above, a rapid, sensitive, and accurate method for the identification of *Legionella* species, and the differentiation of *L. pneumophila* and non-*L. pneumophila* species, becomes extremely important. Here, we pursue a rapid and accurate two-step identification and differentiation scheme to identify and differentiate *L. pneumophila* and non-*L. pneumophila* species. The scheme uses an initial PCR amplification technique for the 16S rRNA gene to identify the *Legionella* species, followed by an enzymatic digestion analysis of the 226-bp fragment of the 16S rRNA gene to differentiate between *L. pneumophila* and non-*L. pneumophila*.

To design a valid PCR, we analyzed the fragments of 16S rRNA genes from 82 *Legionella* strains and performed a theoretical analysis. Based on the results from this analysis, we developed a PCR amplification method to detect all species of the genus *Legionella*.

The differentiation of *L. pneumophila* and non-*L. pneumophila* was achieved by the enzymatic digestion analysis of the 226-bp fragment of the 16S rRNA gene from the PCR assay. This method was based on a finding from our bioinformatics analysis of a 226-bp fragment in the *Legionella* 16S rRNA gene. The bioinformatics data showed that the ACNGT (N = A, G, C, or T) base sequence, identifiable and digestible by HpyCH4III endonuclease, had a remarkably consistent pattern: it appeared at bp 178 to 182 of *L. pneumophila* while non-*L. pneumophila* strains had a variable sequence in this site. If the 226-bp fragment is digested with HpyCH4III endonuclease, the 226-bp PCR products from *L. pneumophila* species will have two fragments with sizes of 180 and 46 bp, while *non-L. pneumophila* isolates will not have them. By running digested samples on agarose electrophoresis, *L. pneumophila* and non-*L. pneumophila* species can be correctly differentiated.

In summary, this two-step assay system is used to detect the genus *Legionella* by PCR amplification and then to differentiate *L. pneumophila* and non-*L. pneumophila* by enzymatic digestion analysis. The performance of this scheme was evaluated with a panel of well-characterized *Legionella* strains obtained from the ATCC and with isolated strains in our laboratory from environmental water samples, such as lakes, pools, rivers, and cooling towers in Guangdong province. The test results by this scheme for the strains mentioned above had remarkable sensitivity and specificity.

MATERIALS AND METHODS

Bacterial strains. Forty-two *Legionella* strains and 12 non-*Legionella* strains were supplied by the American Type Tissue Culture Collection (ATCC) (Table 1). Two hundred *Legionella* strains were from our laboratory. They were isolated from environmental water samples in Guangdong province, and their identities were confirmed by fatty acid analysis, sequence typing analysis, and biochemical analysis. All *Legionella* strains tested were grown on buffered charcoal yeast extract (BCYE) agar and incubated at 37°C in 5% CO_2. The environmental water isolates also were tested by amplified fragment length polymorphism (AFLP) typing or serogroup typing to understand their differences. The serogroup types of the 146 environmental *L. pneumophila* isolates were serogroup 1, serogroup 2, and some undefined serogroups. AFLP types of non-*L. pneumophila* isolates were EWGLI AFLP 013 London, EWGLI AFLP 012 Rome, EWGLI AFLP 014 London, EWGLI AFLP 001 Lugano, EWGLI AFLP 009 London, EWGLI AFLP 019 Dresden, EWGLI AFLP 015 Dresden EUL131, etc. The 54 non-*L. pneumophila* isolates were *L. longbeachae*, *L. sainthelensi*, *L. feeleii*, *L. oakridgensis*, *L. gormanii*, etc.

DNA extraction. Bacterial strains were purified, and nucleic acid extraction was performed with the TIANamp bacterial DNA kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. Each extraction included a negative control sterilized to eliminate the potential contamination of *Legionella* DNA and other environmental contaminants. The extracted sample, ~200 μl in volume containing the genome DNA, was frozen in microcentrifuge tubes at −20°C before analysis.

Bioinformatics analysis for *Legionella* 16S rRNA gene fragment. After searching all of the sequence information for the 386 fragments of 16S rRNA genes of genus *Legionella* from the NCBI (http://www.ncbi.nlm.nih.gov/) nucleotide database, we found that the sequences of the 386 fragments for genus *Legionella* were different from other non-*Legionella* species. At the same time, the sequences within the genus *Legionella* had few variations. We used the fragments from 82 strains, including 8 strains of *L. pneumophila* and 74 strains of non-*L. pneumophila*, for analysis by ClustalX (version 2.0) (1, 20), a bioinformatics software package used to align gene sequences or protein sequences.

PCR assay design. PCR primer sequences were designed for targeting the 226-bp fragment of the 16S rRNA gene for the genus *Legionella*. These 226 bp form the upstream fragment of the total 386 bp. The design mutated site 195 of the 226-bp fragment from T to G. The sequence of the forward primer was 5′-AGGGTTGATAGGTTAAGAGC-3′. The reverse primer was 5′-ATTCCA CTACCCTCTCCCATACTCGAGTCAACC-3′.

PCR assay. Each PCR included 2× *Taq* PCR MasterMix including polymerase, reaction buffer, deoxynucleoside in a ready-to-use formulation, 3 mM $MgCl_2$, 20 mM Tris-HCl (pH 8.3), 100 mM KCl (Tiangen Biotech Co., Ltd). Primers, double-distilled water (ddH_2O), and 4 μl of template were added for a total volume of 50 μl. A GeneAmp PCR system 9700 with a 96-well sample block module (Applied Biosystems) was used for the reaction. The thermal cycling profile consisted of an initial incubation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s, and finally 72°C for 5 min. PCR products were purified by a TIANquick Midi Purification kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions.

HpyCH4III enzymatic digestion analysis assay. The endonuclease of HpyCH4III utilized in the enzymatic digestion analysis of purified PCR products was from New England Biolabs (Neb-China, Beijing, China). Each enzymatic digestion system included 10× NEB buffer 4, ddH_2O, HpyCH4III endonuclease, and 8 μl of purified PCR product for a total volume of 12 μl. Each digestion reaction was carried out at 37°C for 45 min. After the enzymatic digestion, 6 μl of each reaction product was used for agarose electrophoresis analysis.

Primer specificity analysis for *Legionella* 16S rRNA gene. The primer's specificity was analyzed by using the following species as controls: *Salmonella typhi* (ATCC 14028), *Enterobacter cloacae* (ATCC 13047), *Klebsiella pneumoniae* (ATCC 35657), *Haemophilus influenzae* (ATCC 10211), *Proteus mirabilis* (ATCC 35659), *Enterococcus faecalis* (ATCC 29212), *Neisseria meningitides* (WSB 0702), *Pseudomonas aeruginosa* (ATCC 27853), *Yersinia enterocolitica* (WSB 6612), *Stenotrophomonas maltophilia* (ATCC 51331), *Escherichia coli* (ATCC 25922), and *Staphylococcus aureus* (ATCC 6358). These bacterial strains included five bacillus bacterial strains. They were *Salmonella typhi*, *Enterobacter cloacae*, *Kleb-*

TABLE 1. Reference strains used in this study

Species and serogroup	Strain
Legionella strains (n = 42)	
L. pneumophila (n = 16)	
1 (Philadelphia 1)	ATCC 33152
1	ATCC 33153
2 (Togus 1)	ATCC 33154
3 (Bloomington 2)	ATCC 33155
4 (Los Angeles 1)	ATCC 33156
5	ATCC 33216
6 (Chicago 2)	ATCC 33215
7 (Chicago 8)	ATCC 33823
8	ATCC 35096
9	ATCC 35289
10	ATCC 43283
11	ATCC 43130
12	ATCC 43290
13	ATCC 43736
14	ATCC 43703
15	ATCC 33251
Non-*L. pneumophila* (n = 26)	
L. anisa	ATCC 35292
L. adelaidensis	ATCC 49625
L. birminghamiensis	ATCC 43702
L. bozemanae	ATCC 33217
L. fairfieldensis	ATCC 49588
L. cherrii	ATCC 35252
L. cincinnatiensis	ATCC 43753
L. dumoffii	ATCC 33279
L. feeleii	
1	ATCC 35072
2	ATCC 35849
L. nautarum	ATCC 49506
L. parisiensis	ATCC 35299
L. worsleiensis	ATCC 49508
L. jamestowniensis	ATCC35298
L. rubrilucens	ATCC 35304
L. busanensis	ATCC BAA518
L. quinlivanii	ATCC 43830
L. gormanii	ATCC 33342
L. hackeliae	ATCC 35250
L. maceachernii	ATCC 35300
L. longbeachae	ATCC 33462
L. oakridgensis	ATCC 33761
L. wadsworthii	ATCC 33877
L. tucsonensis	ATCC 49180
L. spiritensis	ATCC 35249
L. sainthelensis	ATCC 35248
Non-*Legionella* strains (n = 12)	
Staphylococcus aureus	ATCC 6358
Escherichia coli	ATCC 25922
Yersinia enterocolitica	WSB 6612
Neisseria meningitides	WSB 0702
Salmonella typhi	ATCC 14028
Enterobacter cloacae	ATCC 13047
Klebsiella pneumoniae	ATCC 35657
Haemophilus influenzae	ATCC 10211
Proteus mirabilis	ATCC 35659
Enterococcus faecalis	ATCC 29212
Pseudomonas aeruginosa	ATCC 27853
Stenotrophomonas maltophilia	ATCC 51331

siella pneumoniae, *Haemophilus influenzae*, and *Proteus mirabilis*. The five bacillus bacterial strains utilized in the specificity test of primers were more significant than other bacterial strains, because bacillus strains were closer to genus *Legionella* in evolution than the other bacterial strains. The nucleic acid extraction of these strains was performed under the same conditions as those mentioned above for *Legionella*.

RESULTS

Bioinformatics analysis of *Legionella* 16S rRNA gene fragment. The bioinformatics analysis found that the site at bp 178 to 182 of the 386-bp fragment from the 16S rRNA gene for *L. pneumophila* showed a remarkably consistent pattern: all strains had the base sequence of ACNGT (N = A, G, C, or T), while non-*L. pneumophila* strains had a variable base sequence in this region (Fig. 1). Our bioinformatics analysis also found that some of the non-*L. pneumophila* strains possessed the same base sequence of ACNGT, but it occurred in at bp 151 to 155 or bp 191 to 195, not at bp 178 to 182. The *non-L. pneumophila* strains with ACNGT in the bp 151 to 155 site included *L. birminghamiensis*, *L. brunensis*, *L. donaldsonii*, *L. lansingensis*, *L. quinlivanii*, and *L. spiritensis*. Only a few non-*L. pneumophila* strains had the same base sequence in the bp 191 to 195 site (Table 2).

The finding described above was extremely important, because the base sequence of ACNGT can be digested by HpyCH4III endonuclease. With this in mind, we came up with the novel idea to use HpyCH4III endonuclease to digest the 226-bp fragment, the upstream segment of the 386-bp fragment from the 16S rRNA gene, and then to differentiate *L. pneumophila* and non-*L. pneumophila* by the digested fragment sizes using agarose electrophoresis. The only issue with this design initially was that some of the *non-L. pneumophila* strains with the base sequence of ACNGT in the bp 191 to 195 site would have digested fragments that were too similar to those of the *L. pneumophila* strains. It was difficult to separate them by agarose electrophoresis. To resolve this issue, a novel primer was designed to mutate the 195 site from T to G, which changed the bp 191 to 195 base sequence from ACTGT to ACTGG.

Based on this bioinformatics analysis for all of the *Legionella* species in the NCBI database, we concluded that *L. pneumophila* and non-*L. pneumophila* isolates could be detected and differentiated rapidly by the novel scheme of the PCR assay, followed by enzymatic digestion analysis. The PCR assay was used for identifying the genus *Legionella*. The PCR products of the 226-bp fragment, after it was reacted with HpyCH4III endonuclease, could lead to the specific differentiation of *L. pneumophila* and non-*L. pneumophila*. The key for this scheme was that the 226-bp fragment from *L. pneumophila* strains would be digested to 180- and 46-bp fragments by HpyCH4III, while the 226-bp fragment from a *non-L. pneumophila* strain either could not be digested or was digested with 153- and 73-bp fragments (digested at the bp 151 to 155 site).

PCR optimization and assay results. Primer and template were selected to target the 226-bp fragment of the 16S RNA gene for the genus *Legionella*. This PCR assay was optimized to be simple and versatile. More than 240 genomic DNA samples of the genus *Legionella*, including *L. pneumophila* and non-*L. pneuophila* strains, were analyzed with the PCR. The results for the 226-bp fragments of these samples are shown in Fig. 2a.

Evaluation of primer specificity for the 226-bp fragments of 16S rRNA gene. The goal of this study was to design a method

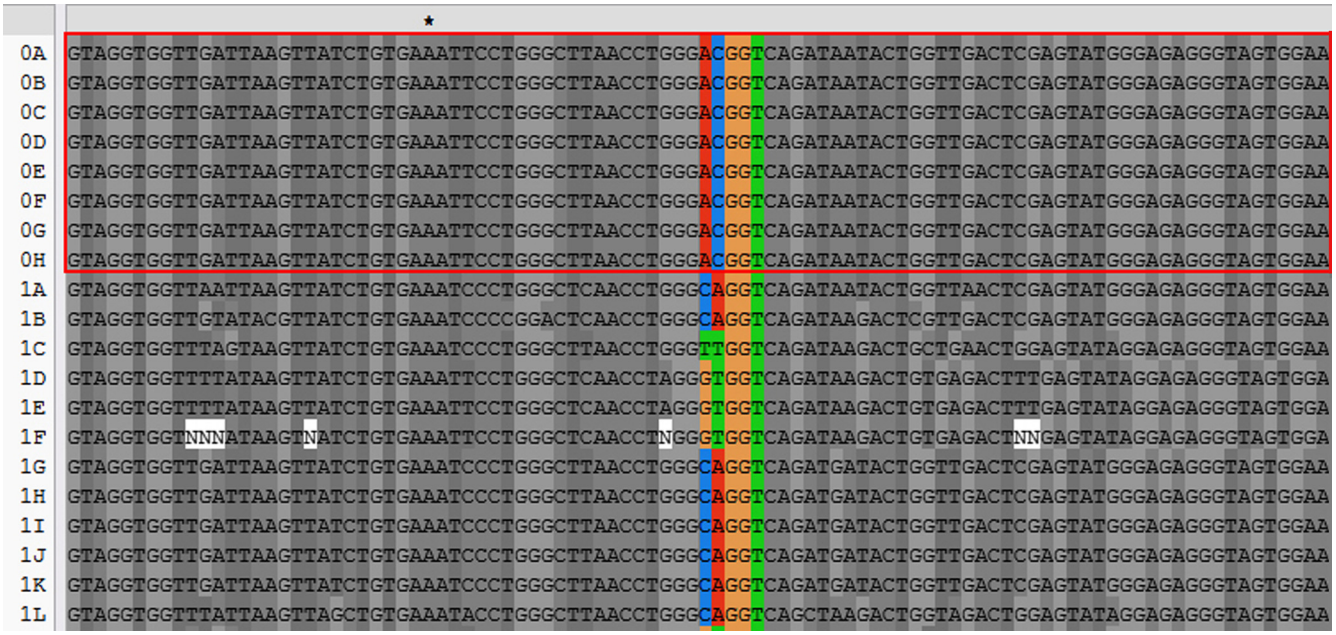

FIG. 1. Bioinformatic analysis of the 386 fragments of 16S rRNA genes from genus *Legionella*. Serial 0 represents the specified sites in *L. pneumophila* isolates. Serial 1 represents the specified sites in non-*L. pneumophila* isolates.

for the rapid detection and identification of *Legionella* species. PCR is considered a rapid method with high sensitivity. However, the specificity of PCR is important in the assay. To determine the specificity of the primer, 12 different strains, including bacillus, Gram-negative, and Gram-positive strains, were used in the experiment. Genomic DNA extraction and PCR methods were performed with the same procedures as those for the *Legionella* strains. The PCR results showed the *Legionella* strains had the 226-bp fragments while all 12 of the other bacterial strains did not have them, even with the annealing temperature lowered to 50°C. (Fig. 3).

Result of HpyCH4III enzymatic digestion analysis. PCR products from *Legionella* strains were purified with a TIANquick Midi Purification kit and then digested by HpyCH4III endonuclease. The digested products were run on agarose electrophoresis to identify fragment sizes.

In this study, 161 strains of *L. pneumophila* and 81 strains of non-*L. pneumophila* were analyzed with the two-step scheme. Among them, 16 strains of *L. pneumophila* and 26 non-*L. pneumophila* species were ATCC reference strains. The other 200 isolated strains were environmental isolates that had been identified to be *L. pneumophila* or non-*L. pneumophila* by fatty acid analysis, biochemical analysis, and sequence typing analysis. The following method was used to differentiate *L. pneumophila* and *non-L. pneumophila*: a strain was *L. pneumophila* if its 226-bp fragment of the 16S rRNA gene was digested to two fragments with sizes of 46 and 180 bp; a strain was non-*L. pneumophila* if its 226-bp fragment either was not digested or was digested to two fragments with sizes of 153 and 73 bp. The experimental results from the reference strains are shown in Table 1. One hundred sixty of 161 strains of *L. pneumophila* had the digested 46- and 180-bp fragments, with only one strain of *L. pneumophila* whose 226-bp fragment was not digested (Fig. 2b), an accuracy of 99.38%. Of the 81 non-*L. pneumophila* species tested by this scheme, all were correctly identified, an accuracy of 100%. Among them, 58 had their 226-bp fragments undigested by HpyCH4III (Fig. 2b); the other 23 strains had their 226-bp fragments digested to two fragments with sizes of 153 and 73 bp. Twenty of these 23 digested strains were *L. feeleii*. The other three were *birminghamiensis*, *L. nautarum*, and *L. quinlivanii*.

TABLE 2. Bioinformatics analysis for *Legionella* 16S rRNA gene fragments

Base sequence	Site (bp)	No. and species
ACGGT	178–182	8 *L. pneumophila*
ACTGT	191–195	29 non-*L. pneumophila*
ACTGT	151–155	7 non-*L. pneumophila*
ACNGT	None	38 non-*L. pneumophila*

Overall, 241 of 242 strains of *L. pneumophila* ($n = 161$) and non-*L. pneumophila* ($n = 81$) were correctly identified by PCR and differentiated by the enzymatic digestion assay, an accuracy of 99.59%. Experiment results for all strains are shown in Tables 3 and 4.

DISCUSSION

Many methodologies have been reported to detect *Legionella* species. These methods, including culture (6), DFA (5, 11), specific PCR (2), and real-time PCR (21), all had their limitations. Another method using *mip* or 16S rRNA gene sequencing was promising because it offered sequence-based identification for *L. pneumophila* and non-*L. pneumophila* species (19, 22), but it was complex and time-consuming. Therefore, a rapid, accurate, and specific method for the identification and differentiation of *L. pneumophila* and non-*L. pneumophila* species is strongly desired.

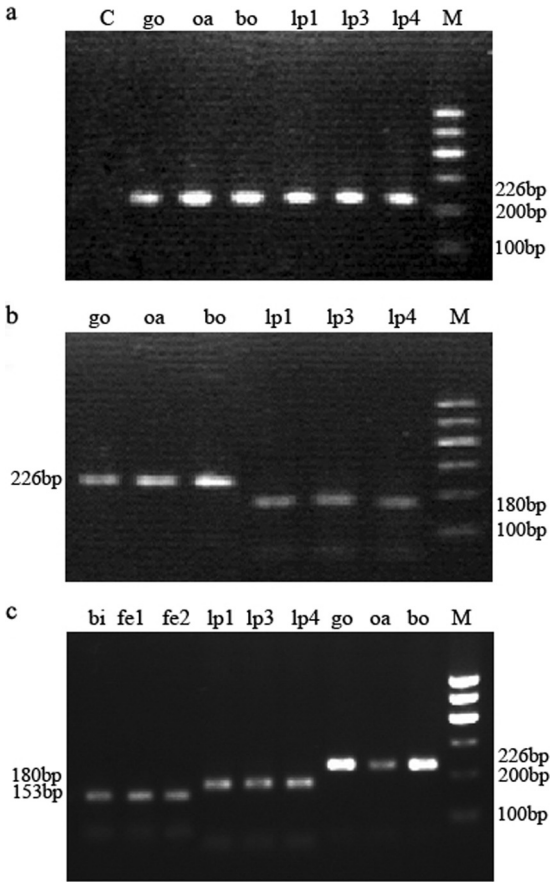

FIG. 2. PCR amplification and HpyCH4III enzymatic digestion analysis of the 226-bp fragments from *Legionella* species. M, 100-bp DNA ladder. (a) PCR amplification for the 226-bp fragments of 16S rRNA genes from *Legionella pneumophila* and non-*L. pneumophila* isolates. (b) HpyCH4III enzymatic digestion analysis of the 226-bp fragments from *L. pneumophila* and non-*L. pneumophila* isolates. The 226-bp fragments from *Legionella pneumophila* all were digested to 180 and 46 bp, but most of the 226-bp fragments from non-*L. pneumophila* species were not digested. (c) Three types of the digestion analysis results: 226-bp fragments from *L. pneumophila* digested to 180 and 46 bp; 226-bp fragments from non-*L. pneumophila* were not digested; and a few 226-bp fragments from non-*L. pneumophila* were digested to 153- and 73-bp fragments. lp1, lp3, and lp4 indicate *L. pneumophila* serogroups 1, 3, and 4, respectively; bi, *L. birminghamiensis*; bo, *L. bozemanae*; *L. gormanii*; oa, *L. oakridgensis*; fe1, *L. feeleii* serogroup 1 (ATCC 35072); fe2, *L. feeleii* serogroup 2.

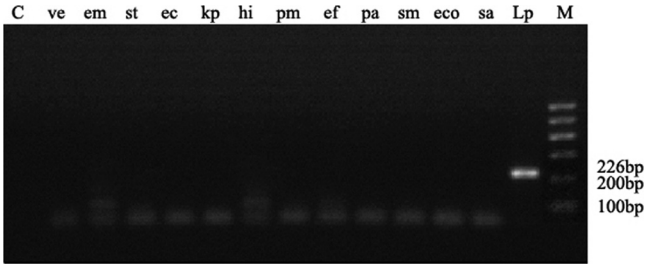

FIG. 3. Evaluation of primer's specificity for 16S rRNA gene 226-bp fragment. M, 100-bp DNA ladder. Non-*Legionella* species are abbreviated as follows: ve, *Yersinia enterocolitica*; nm, *Neisseria meningitides*; st, *Salmonella typhi*; ec, *Enterobacter cloacae*; kp, *Klebsiella pneumoniae*; *hi*, *Haemophilus influenzae*; pm, *Proteus mirabilis*; ef, *Enterococcus faecalis*; pa, *Pseudomonas aeruginosa*; sm, *Stenotrophomonas maltophilia*; eco, *Escherichia coli*; sa, Staphylococcus aureus. Lp is *L. pneumophila*, and C is the negative control.

This study demonstrated a two-step rapid identification scheme for the genus *Legionella* and the differentiation of *Legionella pneumophila* and non-*Legionella pneumophila* species. The scheme is based on a PCR amplification followed by an enzymatic digestion analysis of a 226-bp fragment of the 16S rRNA gene. The PCR product of the 226-bp fragment from the 16S rRNA gene was the key element in discriminating the *Legionella* species from other strains, while the HpyCH4III enzymatically digested fragments of the 226-bp fragment were used to differentiate *L. pneumophila* and non-*L. pneumophila* species. The PCR method was more sensitive and reliable for detecting the genus *Legionella* than DFA and culture (3, 5, 6, 11, 17). The PCR method in this study correctly identified all of the 242 strains of *Legionella*, which included 161 strains of *L. pneumophila* and 81 strains of non-*L. pneumophila* species. The other bacterial strains, including *Salmonella typhi*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, etc., were successfully separated from *Legionella* by this method. The sensitivity of the PCR assay in this scheme was 10^2 CFU (data not shown). The base sequence ACNGT in the 16S rRNA gene of *L. pneumophila*, and the polymorphisms between *L. pneumophila* and non-*L. pneumophila* species, were the essential part of this scheme. This is the first time that this base sequence has been used to differentiate between *L. pneumophila* and non-*L. pneumophila* species. The methods of the sequencing of the *mip* and 16S rRNA genes also utilized gene polymorphisms and interspecies sequence variation to discriminate *L. pneumophila* and non-*L. pneumophila*, but they were time-consuming and thus not practical for clinical purposes.

Our bioinformatics study found the unique nature of polymorphisms for the 226-bp fragment of the 16S rRNA gene between *L. pneumophila* and non-*L. pneumophila*. The 226-bp fragments from the PCR assay for *L. pneumophila* could be digested by HpyCH4III to two fragments with sizes of 180 and 46 bp, while those from non-*L. pneumophila* either would not be digested or would be digested but with two different sizes of fragments (153 and 73 bp). One hundred sixty of 161 *L. pneumophila* strains had their PCR products digested into 180- and 46-bp fragments (Fig. 2b),

TABLE 3. HpyCH4III enzymatic digestion analysis of *Legionella* species and isolates

Strain tested	No. of strains	No. positive by enzymatic digestion for fragment(s):		
		226 bp	180 and 46 bp	153 and 73 bp
ATCC strains	42	22	16	4
L. pneumophila	16	0	16	0
Non-*L. pneumophila*	26	22	0	4
Isolates	200	36	145	19
L. pneumophila	146	1	145	0
Non-*L. pneumophila*	54	35	35	19
Total	242	58	161	23

TABLE 4. HpyCH4III enzymatic digestion results of the 16S rRNA gene and 226-bp fragment for all of the reference *Legionella* strains utilized in this study

Species and serogroup	ATCC no.	Size(s) of fragment(s) after enzymatic digestion (bp)
L. pneumophila		
1	33152	180, 46
1	33153	180, 46
2	33154	180, 46
3	33155	180, 46
4	33156	180, 46
5	33216	180, 46
6	33215	180, 46
7	33823	180, 46
8	35096	180, 46
9	35289	180, 46
10	43283	180, 46
11	43130	180, 46
12	43290	180, 46
13	43736	180, 46
14	43703	180, 46
15	33251	180, 46
Non-*L. pneumophila*		
L. adelaidensis	49625	226
L. bozemanae	33217	226
L. cherrii	35252	226
L. cincinnatiensis	43753	226
L. dumoffii	33279	226
L. parisiensis	35299	226
L. worsleiensis	49508	226
L. jamestowniensis	35298	226
L. rubrilucens	35304	226
L. busanensis	BAA508	226
L. anisa	35292	226
L. gormanii	33342	226
L. hackeliae	33250	226
L. maceachernii	35300	226
L. longbeachae	33462	226
L. oakridgensis	33761	226
L. wadsworthii	33877	226
L. tucsonensis	49180	226
L. spiritensis	35249	226
L. sainthelensis	35248	226
L. quinlivanii	43830	153, 73
L. birminghamiensis	43702	153, 73
L. nautarum	49506	153, 73
L. feeleii		
1	35072	153, 53
2	35849	153, 53

an accuracy of 99.38%. All 81 strains of non-*L. pneumophila* species were successfully discriminated from *L. pneumophila* because their 226-bp fragments either were not digested by HpyCH4III ($n = 58$) or were digested but with fragments of 153 and 73 bp ($n = 23$), an accuracy of 100%.

The specificity of primers for *Legionella* also was evaluated. Twelve different bacterial strains (non-*Legionella* species), including bacillus, Gram-negative, and Gram-positive strains, were used in the evaluation. Species of bacillus were more important controls for the study because they are closer to *Legionella* morphologically and genetically, as many have assumed. The targeted 226-bp fragments were from the 16S rRNA genes that were considered to be an important marker of evolution. The PCR amplification for all 12 of these bacterium strains (5 were bacillus) did not have the targeted 226-bp products, indicating the primer's specificity for *Legionella*.

In conclusion, we have developed a novel two-step scheme for the rapid identification of *Legionella* species and the differentiation of *L. pneumophila* and non-*L. pneumophila* species. This assay system is accurate and takes only about 4 h, whereas the traditional culture method takes 3 to 10 days. Experimental results showed that the two-step method achieved an accuracy of 99.59% (241 of 242). We hope that this convenient two-step scheme can be the choice for the rapid diagnosis of *L. pneumophila* and non-*L. pneumophila* infections, especially for the non-*L. pneumophila* clinical diagnosis.

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