A fast and sensitive real-time PCR assay to detect *Legionella pneumophila* with the ZEN™ double-quenched probe

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Abstract

Legionella pneumophila is a waterborne pathogen that causes respiratory ailments including Pontiac fever and Legionnaires' disease. A culture-free, fast and sensitive detection technique is very important for detection of the pathogen. The present study describes the implementation of rapid cycle real-time PCR in the detection of *Legionella pneumophila* in water through design and development of a real-time qPCR assay based on the ZEN™ probe chemistry. The assay targeted the *mip* gene for the fast and specific detection of *Legionella pneumophila*. The novel assay was very specific and fast as the amplification was obtained within 30 minutes. Sensitivity of the assay as evaluated in terms of its limit of detection (LoD) was as low as 100 cells/reaction with the quantification range between 1×10^3 and 1×10^7 cells/reaction. The assay has been confirmed for repeatability and reproducibility with approximately less than 1% mean intra- and inter-assay variations (CV%). Therefore, the assay reported can be used for a fast, sensitive and specific culture-free detection and quantification of *Legionella pneumophila* in water.

Index Terms: Real time PCR, *Legionella pneumophila*, ZEN™ double-quenched probe, Water borne pathogen, Respiratory pathogen

1. Introduction

Legionella pneumophila (L. pneumophila) – the gram-negative bacterium that naturally occurs as a ubiquitous amoebal parasite in fresh water – may cause sporadic opportunistic infection, mainly in immuno-compromised individuals.[1] The pathogen causes different types of respiratory diseases including an often-fatal pneumonia known as Legionnaires' disease and Pontiac fever.[2] It was first identified as the pathogen that caused the massive outbreak of pneumonia in individuals attending an American Legion convention.[3] Recently, the pathogen has been identified as hosting the largest number of (330+) bacterial effector proteins, which makes it a potential threat to a wide range of hosts including humans.[4]

Conventionally, *L. pneumophila* is detected in water using culture-based methods, which are slow, labor-intensive, costly and create infectious wastes,[5] while giving false negative results due to the fastidious nature of the pathogen. By contrast, real-time PCR provides a faster and more sensitive method of culture-free detection overcoming all these limitations. Real-time PCR has been used to detect and quantify *L. pneumophila* by using TaqMan probes, hybridization probes, molecular beacon probes and EvaGreen dye chemistries.[6-9] However, the minimum time of detection in these assays was 45 minutes.[6]

TaqMan probes are hydrolysis probes with a quencher and a reporter at each end. In its complete form, the TaqMan probe reporter's fluorescence is suppressed by the quencher, but

during PCR amplification the polymerase enzyme hydrolyses the probe that separates the reporter from the quencher, allowing the fluorescence to increase for direct detection of the amplification.

Hybridization probes, on the other hand, are probe systems with two separate oligonucleotides, one with a reporter at the 3'-end and the other with a reporter at the 5'-end. In the annealing step of the PCR amplification, both oligonucleotides anneal to the target sequence, moving the fluorescence reporter and quencher close to each other, and suppressing the fluorescence that allows the detection of the amplification.

Molecular beacons, by contrast, are doubly-labeled hairpin-shaped probe systems. In its hairpin form, the proximity of the reporter and quencher suppresses the fluorescence of the reporter. During amplification, the hairpin structure of the probe opens and hybridizes to the target sequence that separates the reporter from the quencher to allow the detection of the amplification.

Finally, EvaGreen is a double-stranded DNA (dsDNA) binding dye that significantly increases fluorescence upon binding to dsDNA. However, it requires additional post-PCR melting curve analysis, which increases the detection time.

The research reported here takes advantage of recent advancements in real-time PCR thermocycling and detection chemistries to develop a more specific and faster method for detection of the pathogen in water. The 7500 Fast real-time PCR system (Applied Biosystem™ Lifetechnologies, Van Allen Way, U.S.A.) allows rapid cycle amplification and so offers reduced detection time, while the ZEN™ double-quenched hydrolysis probe from IDTDNA, which has an additional internal quencher in between the reporter and quencher, allows for more sensitive and specific detection of target templates.

We also designed novel oligonucleotides based on the highly specific *mip* gene, which encodes a macrophage infectivity potentiator protein virulence factor, to facilitate the entry of the *L. pneumophila* into its hosts,[10,11] thereby enhancing the specificity and selectivity of the assay.

2. Materials and Methods

2.1 Bacterial strains and quantifications

The study used genomic DNA purchased from American Type Culture Collection (ATCC, Manassas, U.S.A.) listed in ***Table 1,*** as a reference strain as well as for cross-reactivity analysis**.** The concentration and purity of the genomic DNA were measured in the NanoPhotometer™ P-Class (Implen, München, Germany) spectrophotometer by reading of the absorbance at 260 nm and by calculating the absorbance A_{260}/A_{280} ratio, respectively. The genomic DNAs were then diluted with 1× Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM disodium EDTA, pH 8.0 to appropriate concentrations before use.

Table 1. Genomic DNA of bacteria stains from ATCC.

Dyes	Stain no./ATCC no.
Legionella pneumophila	ATCC 33152
Staphylococcus aureus	ATCC 25923
Bacillus cereus	ATCC 14579
Bacillus subtilis	ATCC 23857
Salmonella enterica	ATCC 13311
Escherichia coli	ATCC 35401
Clostridium perfringens	ATCC 13124
Shigella flexneri	ATCC 29903
Campylobacter jejuni	ATCC 33292
Yersinia enterocolitica	ATCC 27739
Aeromonas hydrophila	ATCC 7966
Plesiomonas shigelloides	ATCC 51903
Streptococcus pyogens	ATCC 19615
Cronobacter sakazakii	ATCC BAA-894
Mycobacterium avium	ATCC BAA-968

2.2 Primer and probe design

The primer pairs and probes used in this study were designed using the PrimerQuest Tool from Integrated DNA Technologies (IDTDNA, Coralville, U.S.A.) to target a fragment of the *mip* gene of the *L. pneumophila* strain. The primer pairs and probes were designed using sequences of the gene obtained from the GenBank database.[12] The accession number is shown in ***Table 2***.

Primer-Blast[13] was used to confirm the exclusiveness of the primer pairs and probes to *L. pneumophila* strains, while OligoAnalyzer Tool (IDT) assured the absence of strong secondary structures (i.e. primer dimers and hairpin structures) *in-silico*. The primer pair and ZEN™ probe, labeled with the fluorescent dye FAM at the 5'-end, IBFQ quencher at the 3'-end, and an additional internal quencher ZEN™ in the middle of the probe, were purchased from IDTDNA (Singapore Science Park III, Singapore). The oligonucleotides designed and used in this study are listed in ***Table 3***.

2.3 Fast real-time PCR protocol and amplification condition

The assay was carried out on the 7500 Fast real-time PCR system (Applied Biosystem™ Lifetechnologies, Van Allen Way, U.S.A.) in a 25 µL PCR mixture that contained Ultrapure MilliQ water, 1× of Buffer II, 500 nM of both the forward and reverse primers, 250 nM of the probe, 1.5 mM $MgCl_2$, 0.2 mM of dNTP mix (Invitrogen™ Lifetechnologies, Van Allen Way, U.S.A.), 0.1× ROX reference dye (Invitrogen™ Life technologies, Van Allen Way, U.S.A.), 0.625U of AmpliTaq DNA polymerase (Applied Biosystem™ Life technologies, Van Allen Way, U.S.A.) and 4 µL of DNA template, and were run in triplicate or duplicate. Rapid cycle amplification was conducted as follows: Initial denaturation at 95° C for 20 seconds, and 40 cycles of denaturation at 95° C for 3 seconds, Annealing/extension for 30 seconds at 60° C. Negative controls were added for each assay. Negative controls replaced DNA templates with Ultrapure MilliQ water.

2.4 DNA sequencing

The PCR products of the assay were purified and sequenced by First Base Laboratories (Selangor, Malaysia) to determine the specificity of the assay.

2.5 Cross-reactivity analysis

The cross-reactivity of the assay was assessed *in-vitro* with 3×10^6 fg of genomic DNA of bacterial strains as listed in ***Table 1***.

2.6 Sensitivity analysis

The sensitivity of the assay was evaluated by amplifying dilutions of genomic DNA from the *L. pneumophila* reference strain ATCC 3315. The reaction was repeated three times. In the diluted state, 1 cell of *L. pneumophila* was equivalent to approximately 4 fg, calculated from the base pair length of the genomic DNA of the reference strain *L. pneumophila*.[14]

2.7 Validations of assay performance

The performance and quantitative capabilities of the assay were evaluated from the amplification curve prepared by using 10-fold serial dilutions of the genomic DNA of *L. pneumophila* ATCC 33152. The range of concentrations of the dilute solutions was 4×10^2 to 4×10^7 fg/reaction. The experiments were repeated three times.

Table 2. Accession number and location of target gene.

Target gene	Accession no.	Locations	References
mip	AF095230	1-702	Bumbaugh et al., 2002

Table 3. List of designed and selected primer pairs and probes.

Gene	Name		Sequence (5'-3')	Locations	Size (bp)
mip	P1mip	F[a]:	ACATCATTAGCTACAGACAAGG	73-204	131
		P[b]:	6FAM-AGCATTGGT(ZEN)GCCGATTTGGGAAAG -1BFQ		
		R[c]:	CCACTCATAGCGTCTTGC		

[a]: forward primer sequence

[b]: reverse primer sequence

[c]: probe sequence

3. Results and Discussion

3.1 Detection time

In this study, we combined the rapid cycle amplification protocol of 7500 Fast real-time PCR and ZEN™ double-quenched probes to detect *L. pneumophila* in water samples rapidly and reliably. We have chosen the sequence-specific probe-base chemistry (i.e.: the ZEN™ double-quenched probe) to reduce the detection time, as it requires no additional post-amplification melting curve analysis, which could add to the detection time.

The proposed 2-step fast amplification protocol with a fast, real-time PCR instrument reduced the detection time to 30 min, which is significantly faster than conventional culture-based methods that might take up to 10-days,[15] and faster than previously-reported real-time PCR-based methods which use 3-steps standard amplification condition and could take up to 2 hours of amplification time.[6,9,16]

3.2 Specificity of the assay

The specificity of the assay was confirmed with DNA sequencing and cross-reactivity analysis against the bacterial species listed in ***Table 1***. The sequences of PCR products were aligned with the target sequence of *L. pneumophila* ATCC 33152 (accession no. AE017354.1), as shown in ***Figure 1***.

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Query  866922  ACATCATTAGCTACAGACAAGGATAAGTTGTCTTATAGCATTGGTGCCGATTTGGGGAAG  866981
               ||||||| |||| ||| ||||||||||||||| |||||||||||||| ||| |||||||||
Sbjct  1       ACATCAATAGCGTCAGGCAAGGATAAGTTGTTTGATAGCATTGGTGCAGATATGGGGAAG  60

Query  866982  AATTTTAAAAATCAAGGCATAGATGTTAATCCGGAAGCAATGGCTAAAGGCATGCAAGAC  867041
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61      AATTTTAAAAATCAAGGCATAGATGTTAATCCGGAAGCAATGGCTAAAGGCATGCAAGAC  120

Query  867042  GCTATGAGTGG  867052
               ||||||||||||
Sbjct  121     GCTATGAGTGG  131
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Figure 1. Sequence alignment of PCR products of P1mip ZEN™ probe assays with the sequence of *L. pneumophila* ATCC 33152. Query: sequence of *S. aureus* ATCC 25923; Sbjct: sequence of PCR products.

The results demonstrate that the P1mip assay is quite specific and can amplify target sequences. On the other hand, the cross-reactivity analysis establishes that the proposed assay is highly specific to the target species with no cross-reactivity with other species (***Figure 2A***). This is further confirmed by the gel electrophoresis results, which show only a positive amplification band for the positive controls and none for the other bacterial species (***Figure 2B***).

The overall results of the cross-reactivity analysis for the ZEN™ probe P1mip assay are listed in ***Table 4***. This shows that the P1mip assay is highly specific to the target *L. pneumophila* bacteria, without cross-reactivity with other non-*L. pneumophila* bacterial species.

3.3 Limit of detection and assay performance

The sensitivity analysis shows that the P1mip ZEN™ probe assay is able to detect concentrations as low as at 400 fg/reaction or 100 cells/reaction with 77.8% probability. LoD refers to the highest dilutions where observable positive amplification is obtained in the replicates.[17]

Accordingly, the performance of the proposed ZEN™ probe P1mip assay was analyzed by constructing the standard curves and determining the efficiency, the linearity (R^2 value), reproducibility and repeatability (intra- and inter-coefficient variations respectively) of each assay under the proposed fast real-time PCR protocol. The standard curves were generated from 10-fold dilutions of *L. pneumophila* ATCC 33152 genomic DNA that yielded DNA solutions with the concentration range 4×10^2 to 4×10^7 fg/reaction which was equivalent to 1×10^2 to 1×10^7 cell/reaction (see ***Figure 3***).

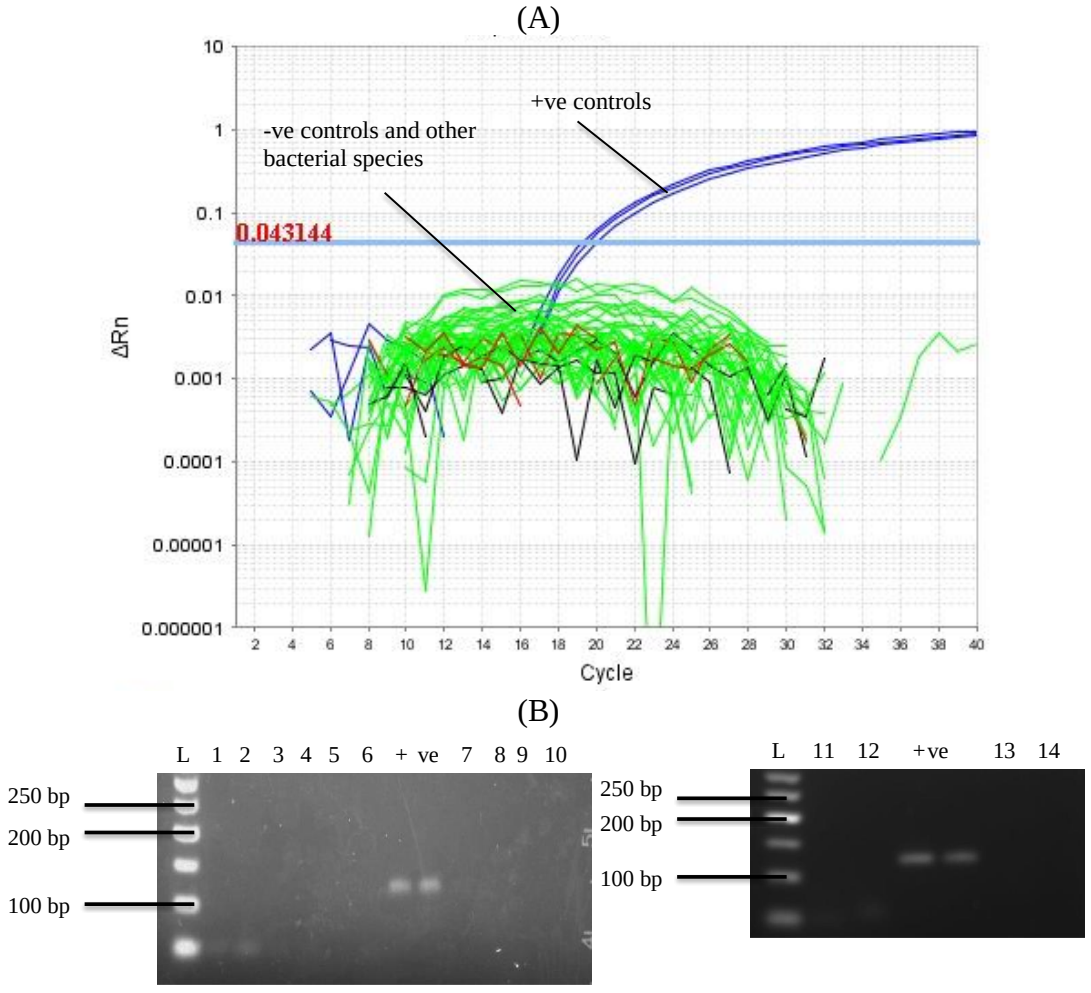

Figure 2. Cross-reactivity analysis for P1mip ZEN™ probe (A) real-time PCR amplification plot; (B) Gel electrophoresis result. (L) 50 bp DNA ladder; (1) *A. hydrophilla;* (2) *B. cereus* (3) *B. subtilis*; (4) *C. jejuni*; (5) *C. perfringens*; (6) *C. sakazakii*; (7) *E. coli*; (8) *M. avium*; (9); *P. shigelloides* (11) *S. flexneri* (12) *S. enterica*; (13) *S. pyogens*; (14) *Y. enterocoalitica*; (+ve) positive control (*L. pneumophila*).

Table 4. Cross-reactivity results of P1mip ZEN™ probe fast real time PCR assays.

Bacterial species	Cross-reactivity results	Ratio of positive reactions[a]
	P1mip	P1mip
L. pneumophila	+[b]	3/3
B. cereus	-[c]	0/3
S. aureus	-[c]	0/3
B. subtlis	-[c]	0/3
S. enterica	-[c]	0/3
E. coli	-[c]	0/3
C. perfringens	-[c]	0/3
S. flexneri	-[c]	0/3
C. jejuni	-[c]	0/3
Y. enterocolitica	-[c]	0/3
A. hydrophila	-[c]	0/3
P. shigelloides	-[c]	0/3
S. pyogens	-[c]	0/3
C. sakazakii	-[c]	0/3
M. avium	-[c]	0/3

[a]: number of positive results per 3 individual reactions

[b]: positive amplifications

[c]: negatuve amplifications

Efficiency was calculated from the standard curve using the equation published by Klein *et al*.[18] The P1mip assays showed an efficiency and linearity (98.923 %; R^2=0.996) within the recommended efficiency range and linearity of 90 to 105 % and R^2>0.99 for real-time PCR.[19] The assay also produced a linear quantification range (see ***Table 5***) of 1×10^3 to 1×10^7 cells/ reaction. The quantification range starts from the highest dilutions with amplifications at >95% probability.[20] It was also highly repeatable, robust, and reproducible and showed approximately less than 1 % mean intra- and inter-assay variation (CV%) (see ***Table 5***). Since the *mip* gene is a single copy gene, the proposed assay can also be used to directly quantify the amount of *L. pneumophila* cell in water samples based on the amount of *mip* gene detected.

4. Conclusion

In conclusion, this study has demonstrated that a faster detection of *L. pneumophila* in water samples is possible using a ZEN™ probe fast real-time PCR assay with amplification in 30 min. However, the quantitative capabilities of the proposed assay with other *Legionella* spp. or *L. pneumophila* contaminated water have not yet

been tested. This will be the subject of future research.

Acknowledgements

Nur Thaqifah Salihah would like to thank the Ministry of Education, Brunei Darussalam for the opportunity to undertake a Ph.D programme at Universiti Brunei Darussalam (UBD).

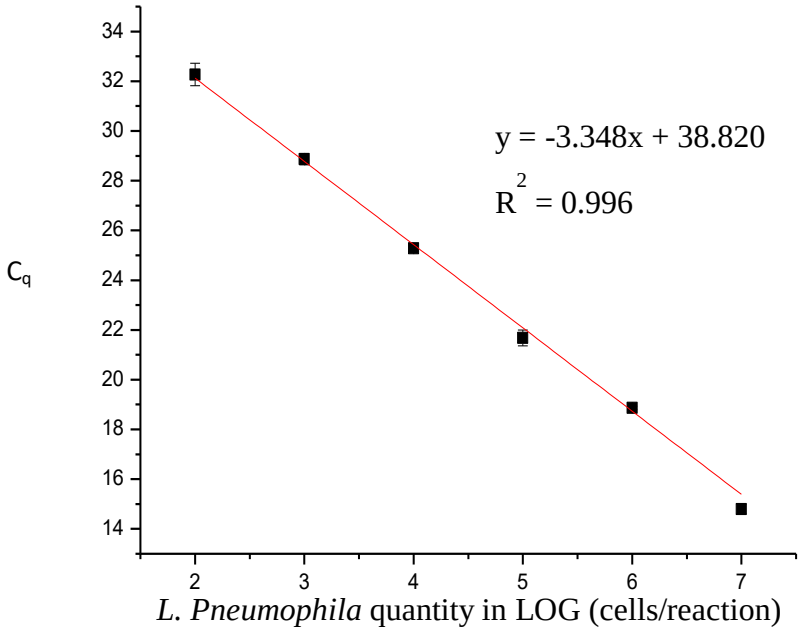

Figure 3. Standard amplification curve for ZEN™ probe P1mip for *L. pneumophila* ATCC 33152 DNA with dilutions between 1×10^2 and 1×10^7 cell/reactions. C_q (the quantification cycle) indicates the number of PCR cycles needed to produce adequate amounts of PCR products to yield a fluorescent signal strong enough to cross the threshold limit for a signal detectable above noise.[5]

Table 5. Ratio of positive reaction and inter- and intra-assay coefficient variation (CV%) for the P1mip real-time PCR assays within the range of 1×10^3 to 1×10^7 cells of *S. aureus* DNA dilution ZEN™ double-quenched probes.

Assay	cell/reaction	Ratio of positive reactions[a]	Mean CV% ± SD[b]	
			Intra-assay	Inter-assay
P1mip	1×10^7	9/9	0.749 ± 0.082	0.699 ±0.349
	1×10^6	9/9		
	1×10^5	9/9		
	1×10^4	9/9		
	1×10^3	9/9		
	1×10^2	6/9		

[a] number of positive result per 9 individual reactions

[b] SD is standard deviations

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