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ORIGINAL ARTICLE

Quick identification and quantification of *Proteus mirabilis* by polymerase chain reaction (PCR) assays

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Abstract *Proteus mirabilis* is an opportunistic pathogen that can cause urinary tract infection in human beings. The accurate and rapid identification and quantification of *P. mirabilis* is necessary for early treatment. In this study, a pair of specific primers according to the conserved *ureR* sequence of *P. mirabilis* was designed and novel systems which consisted of a polymerase chain reaction (PCR) and a real-time PCR to identify and quantify *P. mirabilis* were developed. For the qualitative identification by ordinary PCR, a 225-bp DNA product was amplified from *P. mirabilis* and separated on an agarose gel. The corresponding DNA product is present in three *P. mirabilis* strains isolated from different geographical locations, but is absent in 20 strains representing 18 different species, including the *ureR* homolog contained *Providencia stuartii* and *Escherichia coli* strains, the other common pathogens *Klebsiella* sp., *Edwarsiella* sp., *Vibrio* sp., *Enterobacter* sp., and *Escherichia* sp., and other environmental bacteria *Pseudomonas* sp. and *Acinetobacter* sp. *Proteus mirabilis* at concentrations higher than 1.0×10^3 CFU ml^{-1} was detectable by ordinary PCR; *P. mirabilis* at concentrations higher than 10 CFU ml^{-1} was quantified by real-time PCR. The specific, sensitive and time-efficient PCR methods were demonstrated to be applicable to rapid identification and quantification of *P. mirabilis*.

Keywords *Proteus mirabilis* · *ureR* · Polymerase chain reaction (PCR) · Real-time PCR

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Introduction

Urinary tract infections (UTIs) are among the most frequently occurring human bacterial infections, accounting for about 20 % of all infections acquired outside hospitals. Almost 90 % of UTIs are ascending, with bacteria gaining access to the urinary tract via the urethra to the bladder and then to the upper part of the urinary tract (Hooton 2003; Hryniewicz et al. 2001; Stankowska et al. 2008). *Proteus mirabilis*, a Gram-negative and rod-shaped bacterium, is one of the most common opportunistic pathogens of UTIs in individuals with long-term indwelling catheters or complicated UTIs (Mobley 1996; Mobley and Belas 1995). The traditional methods to detect *P. mirabilis* were to culture this bacterium or amplify its 16S rRNA, followed by biochemical reaction or serological test, and these methods were inconvenient, lowly sensitive and time-consuming (Penner and Hennessy 1980; Suter et al. 1968). Moreover, certain methods may be strongly inaccurate for detection of viable but non-cultivable cells (Colwell 2009). Development of techniques that are specific, sensitive, and time-efficient for identification and quantification of *P. mirabilis* are urgently recommended.

Nowadays, PCR-based methods, in particular quantitative PCR, are used primarily to identify and quantify either

pathogens or beneficial populations, including *Bacillus* sp., *Campylobacter* sp., *Legionella* sp., *Pseudomonas* sp., *Salmonella* sp., and *Vibrio* sp., etc., based on the 16S rRNA genes or their specific functional genes (Amri et al. 2007; Anbazhagan et al. 2010; Brolund et al. 2010; dos Santos et al. 2001; Fiume et al. 2005; Goarant and Merien 2006; Le Dréan et al. 2010; Maligoy et al. 2008; Masco et al. 2007; Mashsouf et al. 2008; Nakano et al. 2003; Postollec et al. 2011; Sauer et al. 2005; Smith and Osborn 2008; Sun et al. 2009; Vanniasinkam et al. 1999; Wehrle et al. 2010). Development of multiple PCR to simultaneously detect common pathogens has also been recommended, such as the PCR methods developed to detect *Enterobacteriaceae* and clinically important bacteria (Cheng et al. 2006; Lu et al. 2000). Species-specific detection of *P. mirabilis* by PCR methods based on 16S rRNA, *ureC* and a function unknown gene have been reported previously (Limanskiĭ et al. 2005; Mansy et al. 1999; Takeuchi et al. 1996). The *ureC*-based PCR method amplified a 533-bp DNA product with a detection limit of 7.0×10^3 CFU ml^{-1} of *P. mirabilis*, and the PCR method based on the function unknown gene amplified a 3,500-bp DNA product with a detection limit of 10 fg DNA of *P. mirabilis*.

ureR of *P. mirabilis* is the only one true regulatory gene that has been identified and is present only in those gene clusters that are inducible by urea. *ureR* of *P. mirabilis* and its close homolog (also designated *ureR*) in the plasmid-encoded urease gene clusters of *Providencia stuartii* and *Escherichia coli* also act as an AraC-like positive activator of gene expression in the presence of urea (Mobley et al. 1995). The *ureR* regulatory gene of *P. mirabilis*, *P. stuartii* and *E. coli* does not have a homolog in the urease gene clusters of *Helicobacter pylori*, *Klebsiella aerogenes*, *Bacillus* sp. or other bacterial species that have been examined thus far (D'Orazio and Collins 1993; Mulvaney and Bremner 1981). Here, by designing a novel pair of specific primers according to *ureR* nucleotide sequence, a *ureR*-based PCR method was introduced to detect *P. mirabilis* for the first time.

In this report, we have developed a *P. mirabilis*-specific ordinary PCR system amplifying part of the *ureR* gene uniquely existed in *P. mirabilis* to qualitatively detect *P. mirabilis* from lake water, seawater and urine. Another real-time PCR system was also developed to quantitatively detect *P. mirabilis* from aquatic environments.

Materials and methods

Bacterial strains and culture media

Three *P. mirabilis* strains and 20 other bacterial strains included in this study are listed in Table 1. All bacterial strains were grown in Luria-Bertani (LB) media (Sambrook et al. 1989) at 28 °C until OD_{600} reached 0.8. The cultures were stored in 50 % glycerol at −80 °C till for use.

Table 1 Bacterial strains used in this study

No.	Strain	Genus and/or species	Source or reference
1	Pd9	*Enterobacter hormaechei*	Isolated from seawater
2	V2	*Enterobacter cloacae*	Isolated from seawater
3	HS51	*Acinetobacter* sp.	Isolated from seawater
4	T32	*Acinetobacter calcoaceticus*	Isolated from seawater
5	Pd2	*Klebsiella* sp.	Isolated from seawater
6	Pd8	*Klebsiella pneumoniae*	Isolated from seawater
7	V134	*Vibrio* sp.	Zhang and Sun 2007
8	T4	*Vibrio harveyi*	Zhang et al. 2008
9	BL	*Vibrio parahaemolyticus*	Isolated from seawater
10	BX	*Vibrio ichthyoenteri*	Isolated from seawater
11	T2	*Edwardsiella tarda*	Isolated from seawater
12	J61	*Pseudomonas* sp.	Isolated from seawater
13	DX7	*Pseudomonas* sp.	Isolated from seawater
14	DJ3	*Pseudomonas plecoglossicida*	Isolated from seawater
15	YK	*Pseudomonas aeruginosa*	Isolated from seawater
16	SP1	*Pseudomonas putida*	Zhang et al. 2011a
17	Ps1	*Providencia stuartii*	GCMCC
18	HS21	*Escherichia* sp.	Isolated from seawater
19	Top10	*Escherichia coli*	Takara
20	HS11	*E. coli*	Isolated from seawater
21	V7	*Proteus mirabilis*	Zhang et al. 2011b
22	Sw11	*P. mirabilis*	Isolated from seawater
23	SY2	*P. mirabilis*	Isolated from crude oil

DNA manipulation

Genomic DNA was extracted from bacterial strains using the DNA extraction kit (Tiangen, China) according to the manufacturer's specifications. DNA concentration was determined using a nanophotometer (Nanodrop, USA). DNA was stored at −20 °C until used for PCR amplification. T4 DNA ligase was purchased from Takara (Dalian, China) and used in accordance with the manufacturer's specifications. When the cloning strategy was employed, PCR products were directly ligated to the cloning vector pBS-T (Takara) and then transformed into the competent cell of *E. coli*. Sequencing was performed by Beijing Genomics Institute (Beijing, China)

Sample collection

Seawater was collected from costal zone (Yantai, Shandong province, China); Lake water was collected from Yantai University; urine of humans was collected when needed. To detect *P. mirabilis* from these samples, 1 μl each original sample or *P. mirabilis*-spiked sample was added into PCR system. To avoid the presence of DNase in urine, the urine

was heated at 95 °C for 30 min, and then heated urine spiked with *P. mirabilis* was used as template of PCR system.

Primers and PCR amplification

The pair of primers was designed using the Primer 5 software according to the *ureR* sequence with accession number Z18752. The forward primer was ureRF1: 5′-GGTGAGATTTGTATTAATGG-3′, and the reverse primer was ureRR1: 5′- ATAATCTGGAAGATGACGAG-3′. Both primers were synthetized by Beijing Genomics Institute.

Each PCR amplification was carried out in a total volume of 15 μl consisting of 1 μl template, 0.5 μl 10 μmol l^{-1} forward primer, 0.5 μl 10 μmol l^{-1} reverse primer, 1.5 μl 10× PCR buffer [200 mmol l^{-1} Tris–HCl (pH 8.4), 200 mmol l^{-1} KCl, 15 mmol l^{-1} $MgCl_2$], 1.5 μl dNTP mix (2.5 mmol l^{-1} each), 0.25 μl Taq DNA polymerase and 9.75 μl distilled water. The amplification conditions were denaturation at 94 °C for 4 min, then 30 cycles of denaturation at 94 °C for 40 s, annealing at 58 °C for 1 min and extension at 72 °C for 20 s, followed by an extension at 72 °C for 10 min. PCR products were electrophoresed on 1 % agarose gel to determine the size of DNA products. Cells of bacterial strains listed in Table 1 were used as templates respectively.

SYBR-green based real-time PCR assay

Real-time PCR was carried out in an ABI 7300 real-time detection system (Applied Biosystems) by using the Sybr ExScript RT-PCR kit (Takara). Dissociation analysis of amplification products was performed at the end of each PCR to confirm that only one PCR product was amplified and detected. PCR mix without template DNA was used as negative control. The amplification conditions were run under the following conditions: each 20 μl reaction mixture consisted of 10 μl SYBR premix, 0.5 μl 10 μmol l^{-1} forward primer, 0.5 μl 10 μmol l^{-1} reverse primer, 0.4 μl ROX reference dye, 1 μl template DNA, and 7.6 μl sterile distilled water. After a denaturation at 94 °C for 30 s, the reaction mixture was run through 40 cycles of denaturation at 94 °C for 5 s, annealing and extension at 58 °C for 20 s, followed by dissociation stage, with 1 cycle of denaturation at 94 °C for 15 s, annealing and extension step at 58 °C for 1 min and then at 94 °C for 15 s.

Results

Viable count of *P. mirabilis* V7

In order to detect the exact cell numbers of *P. mirabilis* V7 contained in one OD_{600}, colony counting experiment was carried out. *Proteus mirabilis* V7 was cultured in LB media to OD_{600} of 0.7, and then the culture was diluted 10^5- and 10^6-fold respectively. One hundred microliters of each dilution was spread on LB agar plates in triplicate. After incubation at 28 °C for 16 h, the colonies that emerged on plates were enumerated. Five hundred, 480, and 442 colonies emerged on the plates when 100 μl of 10^5-fold dilution was spread on plates, and 55, 50, and 49 colonies emerged on plates when 100 μl of 10^6-fold dilution was spread on the plates. Calculation from these data showed that OD_{600} of *P. mirabilis* culture at 1.0 approximately corresponded to 7.1×10^8 CFU ml^{-1} viable cells.

Specificity of *P. mirabilis*-specific PCR

As shown in Fig. 1, using the pair of designed specific primers as forward and reverse primers in PCR amplification, a 225-bp DNA product was amplified from *P. mirabilis*. The DNA product from *P. mirabilis* V7 was cloned into pBS-T and sequenced. The nucleotide sequence of DNA product, amplified by PCR using ureRF1 and ureRR1 as primers and *P. mirabilis* V7 cell as template, is shown in Fig. 2. The sequence showed 100 % similarity to the *ureR* sequence of *P. mirabilis* strain HI4320 with NCBI accession numbers AM942759 and Z18752. The result of the sequence alignment that the *ureR* homologue was found in no other bacterial species but *P. mirabilis* led us to wonder whether *ureR* is a gene unique to *P. mirabilis*. To investigate this speculation, we examined the

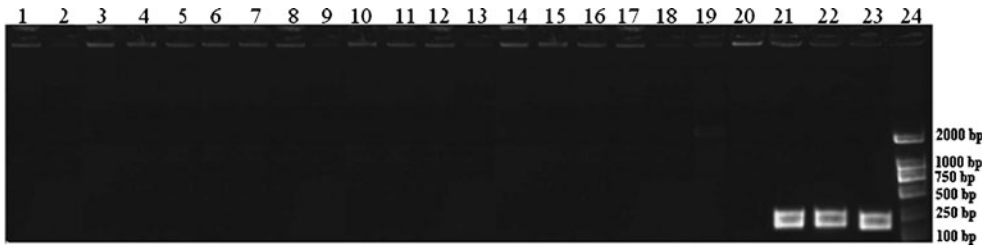

Fig. 1 PCR amplifications using *P. mirabilis* cells and non-*P. mirabilis* cells as templates and ureRF1 and ureRR1 as primers. Templates used were the cells of *E. hormaechei* Pd9 and *E. cloacae* V2 (*lanes 1* and *2*); *Acinetobacter* sp. HS51 and *A. calcoaceticus* T32 (*lanes 3* and *4*); *Klebsiella* sp. Pd2 and *K. pneumoniae* Pd8 (*lanes 5* and *6*); *Vibrio* sp. V134, *V. harveyi* T4, *V. parahaemolyticus* BL, *V. ichthyoenteri* BX (*lanes 7–10*), *E. tarda* T2 (*lane 11*); *Pseudomonas* sp. J61 and DX7, *P. plecoglossicida* DJ3, *P. aeruginosa* YK, *P. putida* SP1 (*lanes 12–16*); *P. stuartii* Ps1 (*lane 17*); *Escherichia* sp. HS21 and *E. coli* Top10 and HS11 *(lanes 18–20)*; *P. mirabilis* V7, SW11 and SY2 (*lanes 21–23*); DNA marker (*lane 24*)

Fig. 2 Nucleotide sequence of the amplified DNA product from *P. mirabilis* V7. The sequence of the primers is *underlined*

GGTGAGATTTGTATTAATGGGCAAACTATCACAGTCACCACTAATCTCACGTTGATTATT
CCTAAATATAGTCAAGTTTCTTGTGATGTGACAAATTTTTTCCCGACCAAACCGATTGAA
TTACATACCTTAGTACTGTCTGAAACTGAATTACAATCTGTGTTCTCTTTACTTAAACCA
TTGATAAAATCAGGGGCACCGATTACTCGTCATCTTCCAGATTAT

prevalence of *ureR* in *P. mirabilis* strains and other different bacterial species collected from different environments. As shown in Fig. 1, the 225-bp DNA product presented in all three *P. mirabilis* strains V7, SW11 and SY2, isolated from differently geographical environments; but no DNA product was observed in the other bacterial strains, including *Acinetobacter* sp., *Klebsiella* sp., *Vibrio* sp., *Enterobacter* sp., *Edwarsiella* sp., and *Pseudomonas* sp., especially the *ureR* homolog contained *P. stuartii* and *E. coli* strains. Together these results demonstrated that using the designed primers, the 225-bp DNA fragment only appeared in PCR product using *P. mirabilis* cell as template, and thus the *ureR*-based PCR method can be used for specific detection of *P. mirabilis*.

Sensitivity of *P. mirabilis*-specific PCR

To determine the sensitivity of the developed *ureR*-based PCR method, *P. mirabilis* V7 was grown in LB media to logarithmic phase and then was 10-fold serially diluted from 1.0×10^7 CFU ml^{-1} to 1.0×10 CFU ml^{-1}. One microlitre of each diluted culture was applied to PCR amplification. As shown in Fig. 3 (lanes 23–29), clear positive DNA bands were observed with concentrations of 1.0×10^3–1.0×10^7 CFU ml^{-1}. A weak positive result (i.e. a faint band) was observed with concentration of 1.0×10^2 CFU ml^{-1}, and this result was not always reproducible.

Detection of *P. mirabilis* from environmental samples

With the above results, we wondered whether the *ureR*-based PCR method could be applicable for detection of *P. mirabilis* from environmental samples. To investigate this speculation, natural seawater, lake water and urine of humans were collected. One microliter of each original sample was used as template for PCR amplification. However, no DNA product was obtained after amplification using natural samples as templates. Natural samples spiked with different *P. mirabilis* concentrations were then used for *P. mirabilis* detection. Results showed that clearly positive DNA bands were observed with concentrations of 1.0×10^3–1.0×10^7 CFU ml^{-1} *P. mirabilis* in lake water (Fig. 3, lanes 16–22); clear positive DNA bands were observed with concentrations of 1.0×10^3–1.0×10^7 CFU ml^{-1} *P. mirabilis* in seawater, but with weaker DNA bands (Fig. 3, lanes 9–15); however, positive DNA bands were observed with concentrations of 1.0×10^4–1.0×10^7 CFU ml^{-1} *P. mirabilis* in urine (Fig. 3, lanes 2–8), and both PCR products obtained using *P. mirabilis* spiked in urine and in heated urine at concentration of 1.0×10^4 CFU ml^{-1} as template showed the same faint DNA bands on agarose gel.

Quantification of *P. mirabilis* by real-time PCR

To detect sensitivity of the *ureR*-based PCR when using DNA as template, serial dilutions of genomic DNA of *P. mirabilis* V7 were applied to real-time PCR. The negative control showed the largest cycle threshold values (Ct), the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level). A linear relationship between DNA input, with DNA concentrations ranging from 85 fg μl^{-1} to 85 ng μl^{-1}, and Ct values was observed with R^2=0.9967 (Fig. 4a). In order to quantify the exact cell number of *P. mirabilis*, 1 ml of 10-fold diluted *P. mirabilis* culture was used to extract genomic DNA, and 1 μl of each extracted DNA was used as template in real-time PCR. Fig. 4b shows that the Ct values had a good linear relationship within a certain cell concentrations ranging from 1.0×10 CFU ml^{-1} to 1.0×10^8 CFU ml^{-1} with R^2= 0.9397. The Ct values had a better linear relationship within cell concentrations ranging from 1.0×10^4 CFU ml^{-1} to 1.0×10^8 CFU ml^{-1} with R^2=0.9905 (Fig. 4c). This result showed that using the *ureR*-based real-time PCR method, *P. mirabilis* concentration at 10 CFU ml^{-1} can be detected and quantified. Higher *P. mirabilis* concentrations, from 1.0×10^4 CFU ml^{-1} to 1.0×10^8 CFU ml^{-1}, was more accurately

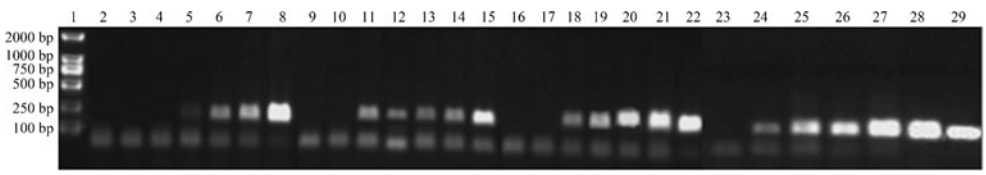

Fig. 3 Sensitivity of *P. mirabilis*-specific PCR. *Lane 1* DNA marker; *lanes 2–8*, *P. mirabilis* spiked in urine at concentrations of 1.0×10^1, 1.0×10^2, 1.0×10^3, 1.0×10^4, 1.0×10^5, 1.0×10^6 and 1.0×10^7 CFU ml^{-1}; *lane 9–15, P. mirabilis* spiked in seawater at concentrations of 1.0×10^1, 1.0×10^2, 1.0×10^3, 1.0×10^4, 1.0×10^5, 1.0×10^6 and 1.0×10^7 CFU ml^{-1}; *lane 16–22, P. mirabilis* spiked in lake water at concentrations of 1.0×10^1, 1.0×10^2, 1.0×10^3, 1.0×10^4, 1.0×10^5, 1.0×10^6 and 1.0×10^7 CFU ml^{-1}; *lane 23–29, P. mirabilis* spiked in ddH2O at concentrations of 1.0×10^1, 1.0×10^2, 1.0×10^3, 1.0×10^4, 1.0×10^5, 1.0×10^6 and 1.0×10^7 CFU ml^{-1}

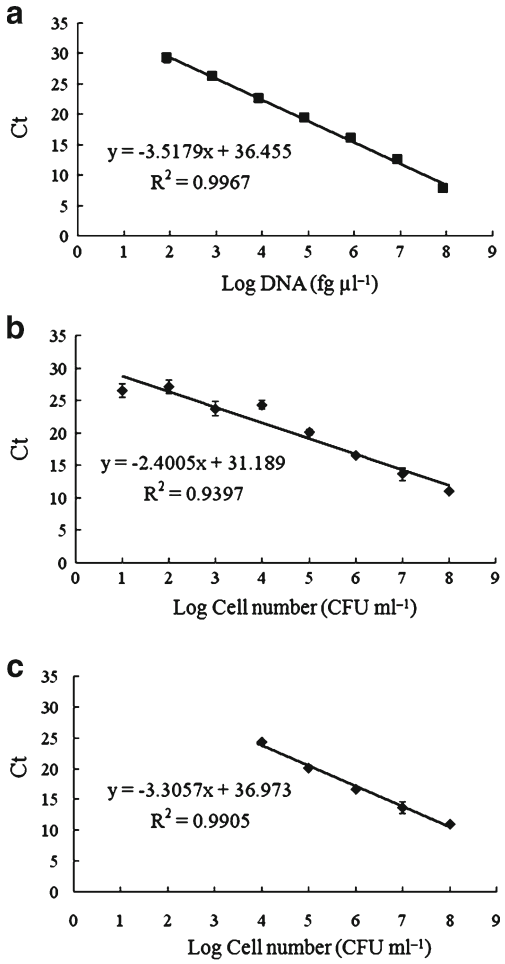

Fig. 4 Relationship between 10-fold serially diluted DNA and Ct value (**a**). A linear range was observed for DNA concentrations from 85 fg μl^{-1} to 85 ng μl^{-1}. Relationship between the serially diluted *P. mirabilis* cells and Ct values obtained (**b**, **c**). *Lines* indicate results of regression analysis (triplicate samples). A linear range was obtained from 1.0×10^{1} CFU ml^{-1} to 1.0×10^{8} CFU ml^{-1} *P. mirabilis* cells (**b**), and a more rigorous linear range was obtained from 1.0×10^{4} CFU ml^{-1} to 1.0×10^{8} CFU ml^{-1} *P. mirabilis* cells (**c**)

quantified through the developed *ureR*-based real-time PCR method.

Discussion

In the last two decades, culture-independent molecular approaches have undergone considerable development in microbial ecology. Compared with culture-based methods, the PCR method is faster, more sensitive and more specific to detect bacterial strains. Moreover, it allows detection of dead cells and viable but non-cultivable cells (Postollec et al. 2011). Several PCR-based methods have been developed to uniquely and rapidly detect *P. mirabilis* specimens. 16S rRNA, *ureC* and a function unknown gene, were employed in previous PCR methods for *P. mirabilis* detection. Compared with the genetic markers 16S rRNA, the housekeeping gene that is preserved in almost all bacterial species, one may speculate that *ureR*-based molecular method should be more specific and discriminating in the detection of *P. mirabilis* (Huang et al. 1999; Limanskiĭ et al. 2005). The result of sequence blast in NCBI that *ureR* uniquely presented in *P. mirabilis* further strengthened this speculation.

For practical reasons, a good diagnostic method should possess the qualities of being sensitive, specific and time-efficient. The *ureR*-based PCR method can be applied directly to crude environmental samples, without, as is required in the previously reported PCR detection methods, cell culturing and DNA preparation. The whole detection process of the *ureR*-based PCR method can be completed in less than 3 h, involving only a PCR amplification and subsequent resolution of the PCR products by electrophoresis in an agarose gel. The comparison between the methods developed previously and the one developed in this study was listed in Table 2. Compared with the PCR method developed by Mansy et al. (1999), the product of *ureR*-based PCR was much smaller and thus it was more time-saving. *ureR*-based PCR detected less bacterial cell numbers than the *ureC*-based PCR method developed by Huang et al. (1999) and Takeuchi et al. (1996) . By ordinary PCR, *P. mirabilis* concentrations higher than 1.0×10^{3} CFU ml^{-1} could be detected from the distilled water, lake water and seawater; however, *P. mirabilis* concentrations higher than 1.0×10^{4} CFU ml^{-1} could be detected from urine. These

Table 2 Comparison of the PCR-based method for the detection of *P. mirabilis*

Primers	Gene	Length (bp)	Detection limit	References
ureC1: 5′ -CCGGAACAGAAGTTGTCGCTGGA- 3′ ureC2: 5′ - GGGCTCTCCTACCGACTTGATC- 3′	*ureC*	533	7×10^{3} CFU ml^{-1}	Huang et al. 1999; Takeuchi et al. 1996
MMKAP1: 5′ - ACCTAGTCCCAGAAAAGAACCCTC- 3′ MMKAP2: 5′ - TGGTGTTAATCACATGCTTGATGG- 3′	Unknown	3,500	10 fg	Mansy et al. 1999
Pr6: 5′ - GGAAACGGTGGCTAATACCGCATAAT- 3′ Pr7: 5′ - GCAGCGCTAGGTGAGCCTAATGGG- 3′	16S rRNA	101	–	Limanskiĭ et al. 2005
ureRF1: 5′ - GGTGAGATTTGTATTAATGG - 3′ ureRR1: 5′- ATAATCTGGAAGATGACGAG – 3′	*ureR*	225	1.0×10^{1} CFU ml^{-1}	This study

features should enable this method to be applicable to various situations often encountered in practical industries, especially to those emergency situations that demand instant diagnosis. The still less DNA product from heated urine excluded the possibility of degrading DNA product by DNase, and thus the less DNA product was probably due to the presence of a PCR inhibitor, such as urea, in urine (Abolmaaty et al. 2007).

The real-time PCR protocol described in this study, including the process of DNA extraction, amplification, dissociation and data collection, can be done within 3 h. Moreover, the *ureR*-based real-time PCR described here can be used for quantitatively detecting *P. mirabilis* DNA from 85 fg μl^{-1} to 85 ng μl^{-1}, and *P. mirabilis* cell number from $1.0\times$ 10 CFU ml^{-1} to 1.0×10^{8} CFU ml^{-1}, respectively. The detection of such a small amount of DNA and small cell numbers may enable the DNA product to be amplified directly from environmental samples and may avoid the extra step of propagating bacteria overnight in culture media. This publication has shown the possibility to follow the growth of *P. mirabilis* in complex environments and has highlighted the potential of molecular approaches in assisting in controlling industrial processes such as has previously been reported (Hagi et al. 2010; Nakayama et al. 2007).

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