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Original Article

Development of TaqMan real-time PCR for detection of *stenotrophomonas maltophilia*

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Abstract: In order to establish efficient and rapid real-time PCR to detect *Stenotrophomonas maltophilia*, specific primers and probe were designed according to the 16SrRNA conservative gene sequence of *S. maltophilia*. Proper TaqMan real-time PCR technique of *S. maltophilia* was developed by optimizing reaction conditions, and performing the sensitivity, specificity, reproducibility test and clinical samples identification. Our results showed the standard had a good linear relationship at the concentration of 1.12×10^{7} to 1.12×10^{2} copies/μL, and the minimum detectable concentration was 1.12×10^{-2} copies/μL, also the method has no direct cross transmission from 21 kinds of bacteria and viruses. The CV values of intra-groups and inter-group is all less than 3%. All tests and clinical samples test showed that the method had many advantages, such as strong specificity, high sensitivity, good stability, high veracity and fast detection. Hence, TaqMan real-time PCR can be used for early diagnosis, rapid detection and quantitative analysis of samples in *S. maltophilia*'s infection.

Keywords: TaqMan real-time PCR, stenotrophomonas maltophilia, detection

Introduction

S. maltophilia, a Gram-negative and non-fermenting bacillus [1], survives widely in soil, water or animal body [2]. It was found in plants for the first time, later, it has become a serious pathogen when it infected humans [3]. *S. matophilia* was classified as *Pseydomonas maltophilia* In 1958, and reclassified as *Xanthomonas maltophilia* in 1983 [4]. Not until 1993, it was identified as the only one character of *Stenotrophomonas spp.* [5]. It can produce a variety of β-lactamases [6], such as *Aminoglycoside-modifying enzymes, Penicillinase and Cephalosporin L2 enzymes*, et al [7]. Hence *S. matophilia* is resistant to *Fluoroquinolones* and *Carbapenms* [8]. Recently, *S. matophilia* is an important opportunistic pathogen, anaerobic bacteria, which was emerging mostly from hospital environment, especially intensive care units (ICU) [9]. *S. matophilia* could always be found from immunosuppressed and debilitated individuals and the patient associated with respiratory infections [10]. On current global trends, the influence of nosocomial *S. matophilia* is increasing substantially and cannot be controlled effectively on account to its mutidrug resistance and decreased membrane permeability [11]. In a long-term study, *S. matophilia* was confirmed to lead to many acute communicable diseases, like malignancy [12], cystic fibrosis and so on. Since it has risen sharply and affected public health, developing an efficient and rapid method to detect *S. matophilia* seems to be particularly pivotal. Up to now, although there are plenty of detection methods that can be applied to test *S. matophilia*, for instance, some traditional methods like identification of bacterial culture and sensitivity test pilot plate, while the general PCR is better than those methods mentioned above. However these methods haven't been widely used due to some of their defects. Consequently, an appropriate TaqMan real-time PCR was established by the means of optimization of PCR conditions, and regarded as a significant tool to detect S. matophilia, thus making beyond compare effects.

Materials and methods

Primer and probe design

The 16S rRNA gene sequences of *S. matophilia* species from GenBank accession, such as No. KU255021.1, KU219842.1 and KU198337.1, et al, were aligned by using DNAMAN. The primers and TaqMan probe in this study were designed by Primer Express 3.0 (Sense primer S.myy-F: GTGAGATGTTGGGTTAAG; Reverse primer S.myy-R: GTCCCTACCATTGTAGTA; probe S.myy-P: CTTGTCCTTAGTTGCCAGCACG). The length of amplification fragment is expected for 174 bp. The primers and probe were synthesized by TaKaRa.

Conditions and standard curve of TaqMan real-time PCR

The *S. matophilia* was purified and cultured on TSA plate, and the Genomic DNA was isolated using TIANamp Bacteria DNA Kit (TIANGEN BIOTECH (BEIJING) Co., LTD.). PCR amplification was conducted in a 50 μL reaction volume with the primer pairs of S.myy-F and S.myy-R. The amplified fragment was purified using Universal DNA Purification Kit (TIANGEN BIOTECH (BEIJING) Co., LTD.) and ligated with pMD19-T (TaKaRa) vector to generate recombinant plasmid and extracted it using TIANprep Mini Plasmid Kit (TIANGEN BIOTECH (BEIJING) Co., LTD.). Afterwards, using the NanoDrop 2000 (Thermo scientific) to measure the concentration of standard before converting it to copy number.

S.myy-F and S.myy-R were applied to target at a 174pb fragment of the cloned region. While S.myy-P was disparate from the conventional PCR. The TaqMan real-time PCR amplification was performed using a Bio-Rad CFX Manager 3.0 (3.0.1224.1015) and the data were analyzed with the CFX Manager™ Software. The standard plasmid was 10-fold serially diluted ranging from 1.12×10^{7}-1.12×10^{1} copies/μL with ddH_2O. Later, the amplification curves and standard curve were established with the aliquot dilutions using CFX Manager™ Software.

Determination of sensitivity

For determining the sensitivity of this assay, the standard plasmid was 10-fold serially diluted ranging from 1.12×10^{7}-1.12×10^{-6} copies/μL, and served as the templates, and the minimum copies were determined under the optimal reaction conditions. Respectively measuring the maximum detectable quantity of TaqMan real-time PCR and general PCR, and the ratio of the two values is the differences of susceptibility.

Test for specificity

Genomic DNA of all the bacterial strains was extracted according to the method mentioned below. The purified cells were cultured in LB or TSB broth that containing 5% bovine serum and 0.002% (w/v) nicotinamide adenine dinucleotide (NAD). The overnight cultures were centrifuged at 8500×g for 5 min, and the cell pellets were resuspended in 200 μL of TE buffer. DNA from these cell pellets were isolated by using TIANamp Bacteria DNA Kit. The DNA of PRV, PCV and PPV were isolated and obtained by conventional approach. These DNA was saved at -20°C as the sample templates of TaqMan real-time PCR for standby application. The 22 species of bacteria and viruses were detected and preserved by Key Laboratory of Animal Disease and Human Health of Sichuan Province from College of Veterinary Medicine of Sichuan Agricultural University.

TaqMan real-time PCR was performed by using different DNA of bacteria and viruses as templates of samples, with S.myy-F, S.myy-R and S.myy-P, at the same time, negative control and blank control were added. Under the optimum reaction condition and system, TaqMan real-time PCR would be approached to evaluate the specificity through its amplification curve.

Evaluation of repeatability

Four dilutions of *S. matophilia* standard were taken as template to start TaqMan real-time PCR, and each dilution repeated four times in one reaction or each reaction repeat four times. Then calculate the coefficient of variation (CV) of intra-group and inter-group according to every value of Ct. CV of intra-group and inter-group can be used for evaluating the stability of TaqMan real-time PCR.

Detection of clinical samples

Twenty parts of tissues were obtained from 20 immunodeficient animals and the clinical samples were detected by two different methods,

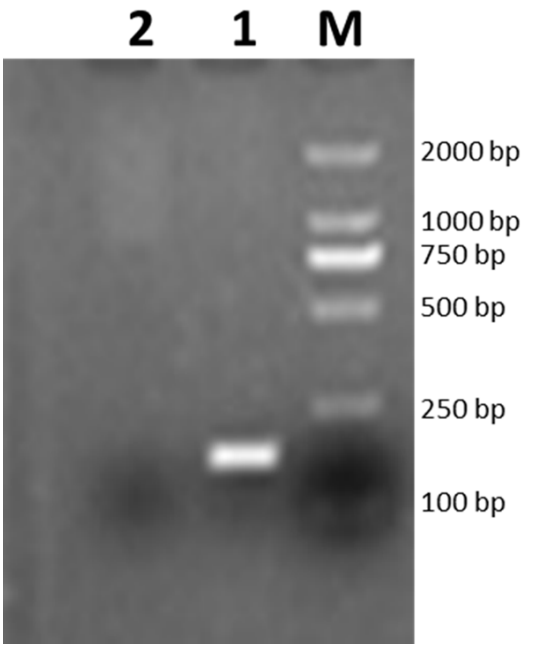

Figure 1. The preparation of standard. PCR identification of recombinant plasmid. M: DL2000 DNA Maker; 1: recombinant plasmid; 2: negative control.

bacterial culture and TaqMan real-time PCR, and then their positive rates calculated. The values were associated with the accuracy between culture method and TaqMan real-time PCR assay.

Results

The preparation of standard

The PCR fragment at a molecular size of 174 bp was obtained using recombinant plasmid along with primer pairs of S.myy-F and S.myy-R (**Figure 1**). With the use of NanoDrop 2000 (Thermo scientific), the recombinant plasmid was measured at the concentration of 35.2 ng/mL, which was converted to copy number at 1.12×10^{10} copies/μL. Subsequently, the recombinant plasmid pMD19-S.myy was prepared as the standard of TaqMan real-time PCR.

Conditions and standard curve of TaqMan real-time PCR

Each reaction mix contained 12.5 μL AceQ® U+ Probe Master Mix (Vazyme Biotech Co., LTD.), 0.5 μL each primer (10 μM), 1 μL probe, 2 μL target DNA and 8.5 μL ddH_2O, with a final reaction volume of 25 μL. PCR cycling program was as follows: 95°C for 30 sec., followed by 40 cycles at 95°C for 5 sec., 55°C for 10 sec., and 72°C for 20 sec.. Negative amplification controls were also included in each run. The standard was diluted with ddH_2O to 10 gradients at 10-fold serial dilutions (1.12×10^{10}-1.12×10^{1} copies/μL). Later, the amplification curves (**Figure 2A**) and standard curve (**Figure 2B**) were established with the aliquot dilutions by using CFX Manager™ Software. It was visible that the standard has a nice linear relationship at the concentration of 1.12×10^{7} to 1.12×10^{2} copies/μL. As was shown in the picture, the amplification efficiency (E), 102.9%, which was close to 100%, meant that the dilution of standard was approximately perfect. Additionally, the correlation coefficient (R^2) was 0.997, manifesting that the concentration of unknown sample can be accurately calculated by the equation below. Consequently, the standard curve, where x represents the logarithm of initial copy number of the template (copies/μL), y represents the value of the Ct of samples.

Sensitivity test

The dilution of standard (1.12×10^{7}-1.12×10^{-6} copies/μL) was served as the templates for sensitivity test. Finally, the minimum for determining was measured as 1.12×10^{-2} copies/μL under the optimal reaction conditions (**Figure 3A**), while the value of general PCR was 1.12×10^{0} copies/μL (**Figure 3B**), what's more, there was no amplified fragment of negative control. So the ratio of the two values was approximately 10^2, meaning that the assay features high sensitivity and accuracy.

Specificity test

Under the optimum reaction condition and system, the result of TaqMan real-time PCR of 22 kinds of different microorganisms shown that there was no amplification curve except for S. matophilia (**Figure 4**). The consequences indicate that the method is highly specific.

Repeatability

According to the table about repeatability test shown below, we known that the CV of intra-group was 1.24%, 2.53%, 1.23%, 0.51% (**Table 1**) and the CV of inter-group was 2.04%, 2.75%, 0.96%, 0.59% (**Table 2**), respectively. All of them were less than 3%. That means the

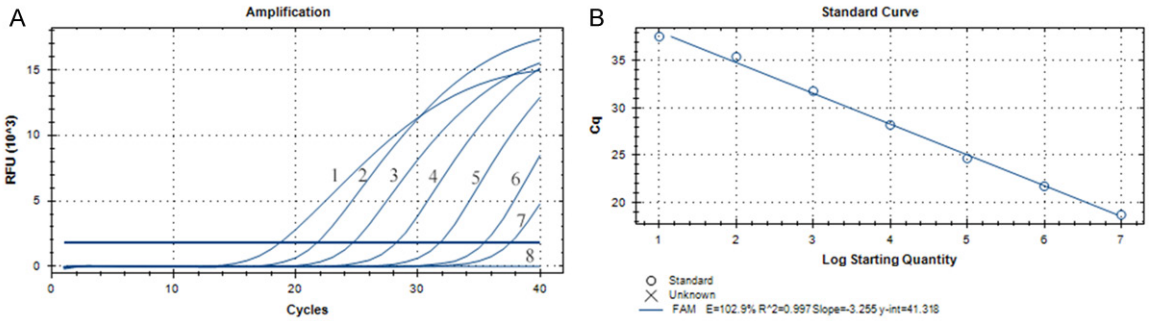

Figure 2. Conditions and standard curve of TaqMan real-time PCR. A. Amplification curves of different dilutions of standards of TaqMan real-time PCR. 1-7: The recombinant plasmid pMD19-S.myy dilution from 1.12×10^{7}-1.12×10^{1} copies/μL, respectively; 8: negative control. B. Standards curve of real-time PCR.

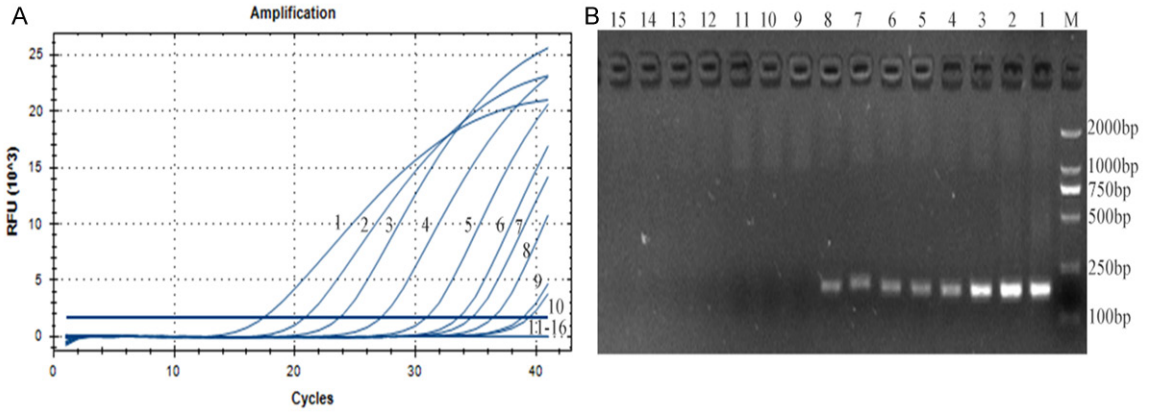

Figure 3. Sensitivity test. A. Sensitivity test of TaqMan real-time PCR for the recombinant plasmid pMD19-S.myy. 1-14: pMD19-S.myy dilution from 1.12×10^{7} to 1.12×10^{-6} copies/μL, respectively; 15: negative control; 16: blank control. B. Sensitivity test of the conventional PCR for pMD19-S.myy. 1-14: pMD19-S.myy dilution from 1.12×10^{7} to 1.12×10^{-6} copies/μL, respectively; 15: negative control.

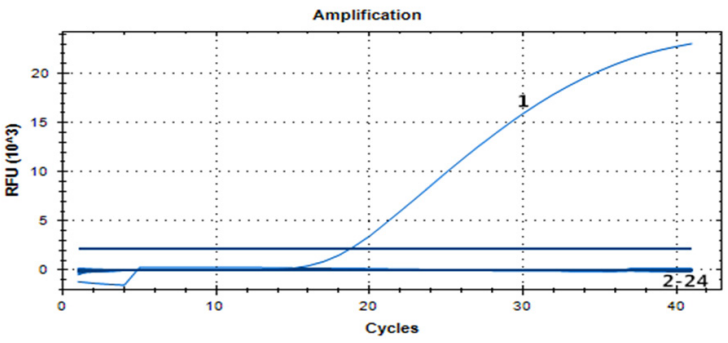

Figure 4. Specificity test of real-time PCR. Specificity test of TaqMan real-time PCR. 1: S. maltophilia, 2: Erysipelothrix rhusiopathiae, 3: Proteus mirabilis, 4: Haemophilus parasuis, 5: Acinetobacter baumannii, 6: Pasteurella multocida, 7: Salmonella, 8: Escherichia coli, 9: Actinobacillus pleuropneumoniae, 10: Staphylococcus aureus, 11: Streptococcus uberis, 12: M.tuberculosis, 13: Bacillus anthraci, 14: Clostridium tetani, 15: Clostridium welchii, 16: Klebsiella pneumonia, 17: Chlamydia, 18: M.Pneumonia, 19: pseudomonas aeruginosa; 20: PRV, 21: PCV, 22: PPV; 23: negative control; 24: blank control.

TaqMan real-time PCR of *S. maltophilia* has high stability.

Detection of clinical samples

Based on experimentation of 20 samples, **Table 3** shown the positive rates of TaqMan real-time PCR was 55% (11/20) (**Table 3**), which was same to the results of bacterial culture (**Figure 5**). As well as, it has one and only 11 amplification curves when proceeding TaqMan real-time PCR. The test indicated that TaqMan real-time PCR was a rapid and simple assay with high credibility for the positive incidence of the samples between the two is equal.

Table 1. The reproducibility tests of real-time PCR about intra-group

NUMBER	Ct1	Ct2	Ct3	Ct4	SD	AVE	CV
No.1	17.12	17.46	17.39	17.01	0.21	17.25	1.24%
No.2	21.58	20.96	20.85	20.29	0.53	20.92	2.53%
No.3	23.93	24.31	23.78	23.62	0.30	23.91	1.23%
No.4	26.61	26.87	26.77	26.58	0.14	26.71	0.51%

Table 2. The reproducibility tests of real-time PCR about inter-groups

NUMBER	Ct1	Ct2	Ct3	Ct4	SD	AVE	CV
No.1	17.85	17.17	17.05	17.28	0.35	17.34	2.04%
No.2	23.83	23.06	22.28	23.11	0.63	23.07	2.75%
No.3	20.29	20.36	20.21	20.66	0.20	20.38	0.96%
No.4	26.08	26.03	26.20	26.38	0.16	26.17	0.59%

Table 3. The identification tests of real-time PCR about clinical samples

Well	Fluor	Content	Cq	Cq Mean	Cq Std. Dev
A01	FAM	Pos Ctrl	17.36	17.36	0.000
A02	FAM	Unkn	N/A	0.00	0.000
A03	FAM	Unkn	N/A	0.00	0.000
B01	FAM	Unkn	N/A	0.00	0.000
B02	FAM	Unkn	N/A	0.00	0.000
B03	FAM	Unkn	32.17	32.17	0.000
C01	FAM	Unkn	24.89	24.89	0.000
C02	FAM	Unkn	36.37	36.37	0.000
C03	FAM	Unkn	8.82	8.82	0.000
D01	FAM	Unkn	34.25	34.25	0.000
D02	FAM	Unkn	N/A	0.00	0.000
D03	FAM	Unkn	N/A	0.00	0.000
E01	FAM	Unkn	9.13	9.13	0.000
E02	FAM	Unkn	31.96	31.96	0.000
E03	FAM	Unkn	N/A	0.00	0.000
F01	FAM	Unkn	N/A	0.00	0.000
F02	FAM	Unkn	17.34	17.34	0.000
F03	FAM	Neg Ctrl	N/A	0.00	0.000
G01	FAM	Unkn	34.88	34.88	0.000
G02	FAM	Unkn	11.01	11.01	0.000
G03	FAM	NTC	N/A	0.00	0.000
H01	FAM	Unkn	29.37	29.37	0.000
H02	FAM	Unkn	N/A	0.00	0.000

Discussion

S. maltophilia, a nosocomial opportunistic pathogen, is considered as a prototype of intrinsically resistant bacterium and widely exists in animals [13]. Its separation rate has been rising gradually [14], just after *Pseudomonas aeruginosa* and *Acinetobacter baumann* [15], for to the extensive use of antibiotics and immunosuppressant. *S. maltophilia* brings a great deal of difficulties to clinical treatment for its drug efflux pumps [16] of multiple antibiotics and a quinolone resistance protein [17]. *S. maltophilia* mainly induces respiratory infections and pneumonia in immunocompromised hosts [18] (including humans and animals). Even more, It is extraordinarily fatal that *S. maltophilia* shows high mortality and indicates the ability to frequently cause underlying diseases, especially when infecting chronically colonized cystic fibrosis patients [19] and debilitated individuals [20]. Little is known about *S. maltophilia*, however, the rising of the separation rate about *S. maltophilia* of clinical isolation makes it tough to identify pathogens [21].

In the research, the primers and probe was designed by aligning the gene sequences of 16SrRNA, selected from GenBank, about S. maltophilia. With the help of NCBI Primer BLAST tool, we found that there is no non-specific amplification in pig, humans, and bacteria. At least, the result ensured the specificity of the primers in theory. The TaqMan real-time PCR was established to detect S. maltophilia by optimizing reaction system (25 μL) and conditions (Tm=55°C), ensuring the veracity and reliability of a series of experiments below. The specificity test showed excellently specificity as there was not any amplification in 21 samples excepted for a S. maltophilia. That meant the assay can be used for rapid, precise and quantitative detection of clinical samples. In addition, the TaqMan real-time PCR of S. maltophilia had a good linear relationship when the standard copy number ranged from 1.12×10^{7} copies/μL to 1.12×10^{1} copies/μL. From the linear relationship, we found E=102.9%, extraordinarily close to 100%, meaning that the gradients dilution of standard was approximately perfect and the amplification curves were credible. In addition, R^2 was 0.997, indicating that the equation, could be applied to calculate the concentration of clinical sample and the result must be extremely precise. Furthermore, the lowest concentration of pMD19-S.myy it could detect was 1.12×10^{-2} copies/μL, which mani-

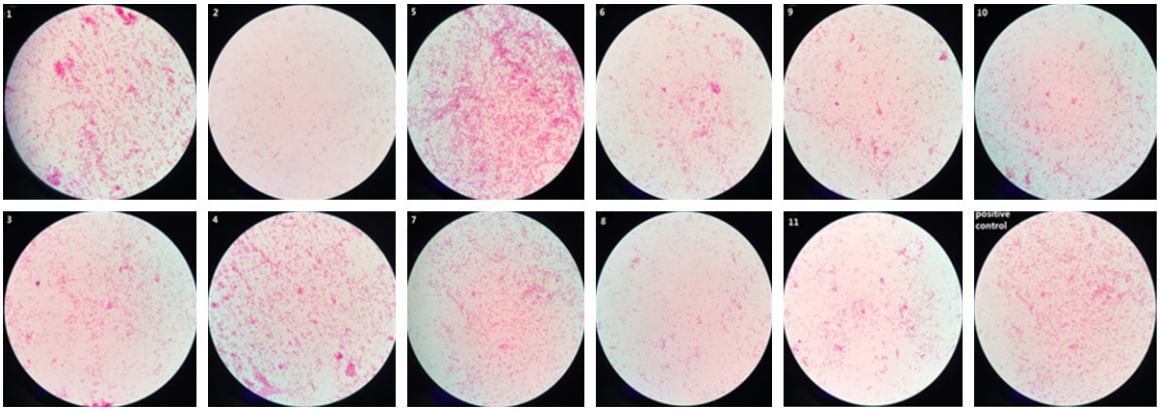

Figure 5. The pictures of microscopic examination of the 11 kinds of clinical samples and positive control and positive control.

fested this TaqMan real-time PCR assay of S. maltophilia could be used for effectively measuring its concentration, even the content was very low. By our results, we known the coefficient of variation (CV) of intra-group and intergroup were both less than 3%. It indicated the design of this experiment was reasonable, and the assay had altitudinal sensitivity and reproducibility when using it to detect *S. maltophilia*. Although, there has reported a lot of identification technologies about *S. maltophilia*, such as bacterial culture and general PCR, these assays has never been used widely due to their defects in accuracy, sensitivity or specificity. While, the general PCR was more insensitive when it was compared to SYBR Green qPCR [22], yet, the sensitivity of SYBR Green qPCR was lower than TaqMan real-time PCR. According to the research, the results demonstrated that the susceptibility of TaqMan real-time PCR was nearly 10^2 times higher than general PCR, signifying it is a promising technology with high sensitivity for detecting *S. maltophilia*. The experimental results of the assay is satisfying, even when the concentration is very low, that means the method is very suitable for low concentration detection of *S. maltophilia*. In the detection of clinical samples, we prepared 20 specimens of pigs for TaqMan real-time PCR and bacteria culture. It was exhibited that detection of 11 samples and positive control shown as positive results, while negative control and the other 9 samples did not perform any nonspecific amplification curve. It indicates that the *S. maltophilia* claims our highest attention for the result shows its separating rate is high, and it can survive in various kinds of situations. In addition, the experimental data of TaqMan real-time PCR was consistent with bacteria culture. It demonstrated that the almost unprecedented advanced technology of *S. maltophilia* was deemed feasible and trustworthy.

On the basis of our investigation, we realized, at least in China, there was no paper about SYBR Green qPCR for testing *S. maltophilia* has been reported, not to mention TaqMan real-time PCR. Meanwhile, the development of TaqMan real-time PCR of *S. maltophilia* anywhere else is extraordinarily immature. What's more, with the impact and danger of *S. maltophilia* is growing sharply, the detection of it should be taken into widely consideration. Without any question, in view of the importance of this issue and the above-mentioned facts, the TaqMan real-time fluorescent quantitative PCR basing ordinary PCR is a new technique for detecting *S. maltophilia* precisely, fleetly and quantitatively, which possess characteristic of sensitiveness, quickness and specialty, and can accurately fix the quality. We propose a detailed model for TaqMan real-time PCR as a measurement of real-time variations of amount to provide an unprecedented advanced technology of *S. maltophilia*, at least in China, in terms of the fluorescence signal changes.

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Disclosure of conflict of interest

None.

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