Scientific Research

Development of a Duplex Real-Time PCR Method for the Pharmaceutical Rapid Microbial Detection of *Staphylococcus aureus* and *Pseudomonas aeruginosa*

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Abstract

Objective: To develop a duplex real-time PCR assay for pharmaceutical rapid microbial detection of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Methods: The specific primers and probes were designed to amplify the *femB* gene of *S. aureus* and the *DNA gyrase subunit B* gene of *P. aeruginosa*. The sensitivity of the system was detected by a multiple proportional dilution method. In order to examine the specificity of the system, other twenty-one bacteria strains were assayed simultaneously. Results: A highly sensitive and specific duplex real-time PCR assay for the detection of *S. aureus* and *P. aeruginosa* was established. The sensitivity was 50 copies/μL. The specificity was 100%. The whole detection procedure can be finished within 2.5 h. Conclusion: The duplex real-time PCR method is efficient in detecting with good sensitivity and specificity. There is a good prospect of this method applying in disease prevention and pharmaceutical industry due to the simultaneous detection of two pathogens.

Keywords

S. aureus, P. aeruginosa, Duplex Real-Time PCR, Pharmaceutical

1. Introduction

S. aureus is one of the most adaptable human pathogens causing a wide variety of diseases in humans ranging from localized skin and soft-tissue infections to more serious disease due to disseminated infection including septicemia, pneumonia, endocraditis, deep-seated tissue abscesses, osteomyelitis and meningitis [1]. *P. aerugi-*

nosa is an opportunistic pathogen in humans and a major cause of morbidity and mortality in patients with cystic fibrosis (CF) [2] [3].

Real-time PCR assays based on platforms such as the ABI 7500 sequence detection system, more commonly referred to as the Taqman, have steadily become more established for the detection and identification of a variety of organisms including clinically important bacteria such as *C. jejuni* [4] [5], *Listeria monocytogenes* [6] and *Salmonella species* [7]. The Taqman system exploits the 5' nuclease activity of *Taq* DNA polymerase in conjunction with fluorogenic DNA probes. The probe is designed to hybridise an internal region of the PCR product and is labelled at the 5' end with a fluorescent reporter dye and at the 3' end with a quencher dye. The intact probe is of sufficient length that, under normal conditions, the fluorescence of the reporter is suppressed due to its spatial proximity to the quencher. However, during PCR amplification the hybridised probe is hydrolysed by the 5' nuclease activity of *Taq* polymerase, thus separating the reporter dye from the quencher. An increase in reporter dye fluorescence is a direct result of target amplification. Repeated PCR cycles result in an increase in fluorescence corresponding to amplification of the target PCR product, which is detected and recorded by the system.

The use of real-time PCR (RT-PCR) allows turnaround time and eh risk of contamination with PCR products to be reduced compared to the conventional PCR method. In the present study, a highly sensitive and specific duplex real-time PCR assay for the detection of *S. aureus* and *P. aeruginosa* was established. The sensitivity was 50 copies/μL. The specificity was 100%. The whole detection procedure can be finished within 2.5 h.

2. Material and Methods

2.1. Bacterial Strains

Pseudomonas aeruginosa [CMCC(B)10104], *Staphylococcus aureus* [CMCC(B)26003], *Staphylococcus epidermidis* [CMCC(B)26069], *Shigella flexneri* (ATCC 12022), *Listeria monocytogenes* (GIM 1228), *Vibrio parahaemolyticus* (ATCC 17802), *Escherichia coli O157* (NCTC 12900), *Shigella sonnei* [CMCC(B)51592], *Enterococcus faecalis* (ATCC 29212), *Aeromonas hydrophila/caviae* (GIMI 172), *Yersinia enterocolitica group* [CMCC(B)52204], *Bacillus cereus* [CMCC(B)63303], *Bacillus subtilis* [CMCC(B)63501], *Escherichia coli* [CMCC(B)44102], *Salmonella* [CMCC(B)50094], *Streptococcus sanguinis* [CMCC(B)32210], from Chinese food and Drug Inspection Institute, *Listeria ivanovii* (ATCC 19119), *Listeria innocua* (ATCC 33090) donated from Guangdong provincial center for disease control and prevention.

2.2. Primers and Probes

Primer and probes sets were designed for *S. aureus* gene *fem B* and *P. aeruginosa* gene *DNA gyrase subunit B* using the criteria within Primer Express (Applied Biosystems version 1.5). All primers and probes were checked in BLASTN to ensure they were unlikely to amplify other genes from other species and organisms (Table 1).

2.3. DNA Extraction

Bacterial DNA extraction was carried out using Promega DNA extraction kit. The rest of the procedures were as recommended by the manufacturer. The concentration and purity of the extracted DNA was determined by measuring the absorbance ratio at wavelength 260 nm over 280 nm using a spectrophotometer.

2.4. Duplex Real-Time PCR

Each DNA extraction were tested in duplicate and were amplified using Roche LightCycler 480 real-time PCR

Table 1. Target genes and primer and probe sequences used for duplex real-time PCR.

Gene	Forward primer	Reverse primer	Probe
Fem B	AGGTCGCGAGAAAAATGATG	TACGCCCATCCATCGTACTT	FAM-TGGTTACGAGCATCATGGCTTTACAA-BHQ1
DNA gyrase subunit B	TACCTGAACACCAACAAGACCG	CTTCGGCCTCGATGTAGTTGTT	HEX-AACGACAGCTTCAACGAGAACCTGCT-BHQ1

detection system with an initial hold at 95˚C for 10 min followed by 45 cycles at 95˚C for 10 s sand 58˚C for 45 s, to determine the best primer working concentration and probe working concentration.

2.5. Assay Detection Limit

The detection limit of the Duplex real-time PCR assay was determined using 10-fold serial dilution of DNA extraction ranging from 105 to 10 copies DNA/μL, and the real-time PCR detection was carried out to establish standard curve. Three separate real-time PCR were assayed to determine the detection limit.

2.6. Assay Specificity

For testing specificity, 23 unrelated microorganism isolates, including *S. epidermidis*, *Shigella flexneri*, *L. monocytogenes*, *V. parahaemolyticus*, *E. coli O* 157, *Shigella sonnei*, *E. faecalis*, *A. hydrophila/caviae*, *Y. enterocolitica*, *B. cereus*, *B. subtilis*, *E. coli*, *Salmonella*, *S. sanguinis*, *L. ivanovii*, *L. innocua* and 4 environmental isolates, were used to confirm specificity of the assay.

2.7. Assay Reproducibility

Four duplicable tests were assayed within a RT-PCR run to determine the mean, standard deviation (S.D.) and coefficient of variation (CV).

3. Results

3.1. Optimization of the Real-Time PCR System

Optimization of the assay was carried out to assess the best combination of probes and primers for maximum reaction efficiency, and the reaction volume was reduced by half to lower costs, without compromising specificity.

3.1.1. The Best Primer Working Concentration

The probe concentration was set to 200 nM, and the PCR assay was carried out to choose the best primer working concentration, which was showed in **Table 2**.

The concentration of the curve with the minimal Ct value and obvious amplification curve was choose as the best primer working concentration, which were showed in **Figure 1**, **Figure 2**. That is Forward primer 400 nM and Reverse primer 400 nM.

3.1.2. The Best Probe Working Concentration

The Forward primer and Reverse primer concentration was set to 400 nM, and the real-time PCR were carried out to choose the best probe working concentration from 100 nM to 800 nM.

The concentration of the curve with the minimal Ct value and obvious amplification curve was choose as the best probe working concentration, which were showed in **Figure 3**, **Figure 4**. That is 200 nM.

3.2. Assay Sensitivity

The detection limit of the Duplex real-time PCR assay was determined using 10-fold serial dilution of DNA

Table 2. The best primer working concentration (*S. aureus* and *P. aeruginosa*).

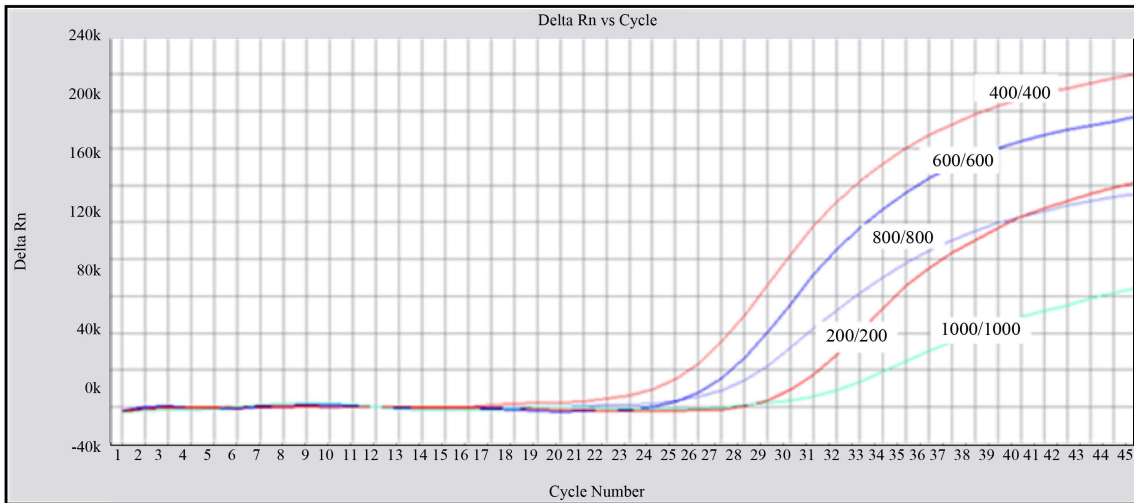|		*P. aeruginosa*/*S. aureus*-RP (concentration nM)				
		200	400	600	800	1000
	200	200/200	200/400	200/600	200/800	200/1000
	400	400/200	400/400	400/600	400/800	400/1000
P. aeruginosa/*S. aureus*-FP (concentration nM)	600	600/200	600/400	600/600	600/800	600/1000
	800	800/200	800/400	800/600	800/800	800/1000
	1000	1000/200	1000/400	1000/600	1000/800	1000/1000

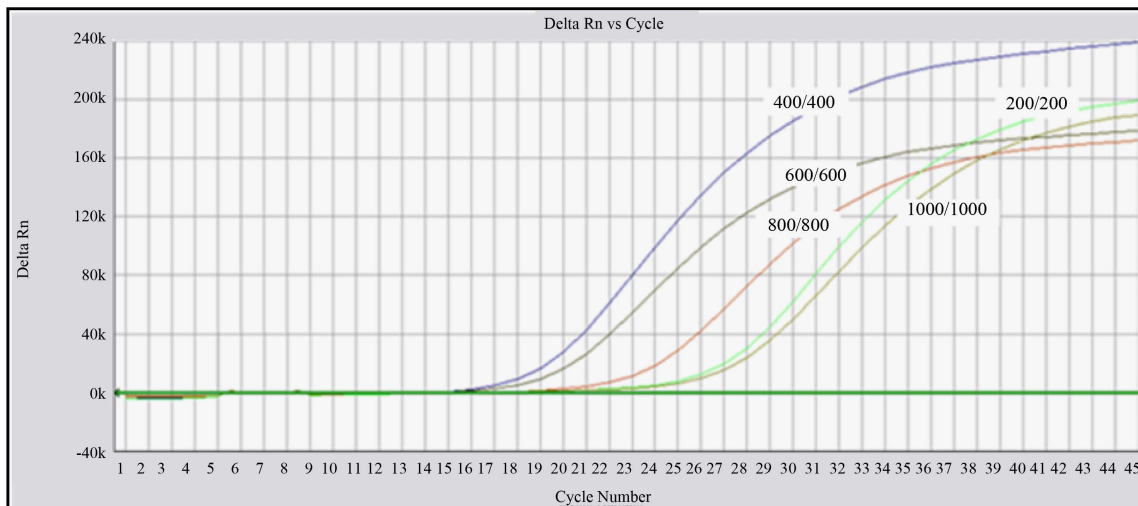**Figure 1.** The best primer working concentration (*S. aureus*).

Figure 2. The best primer working concentration (*P. aeruginosa*).
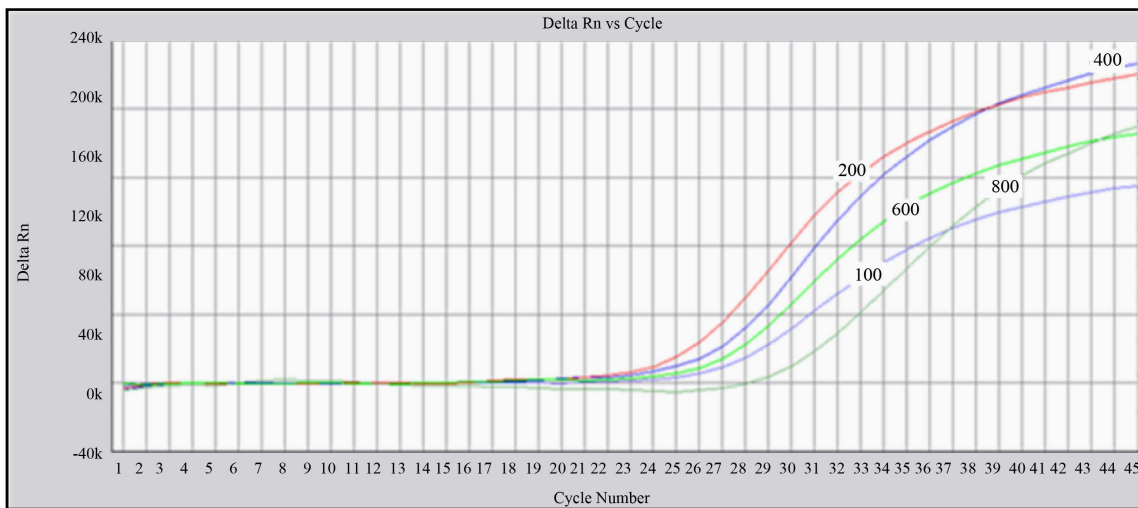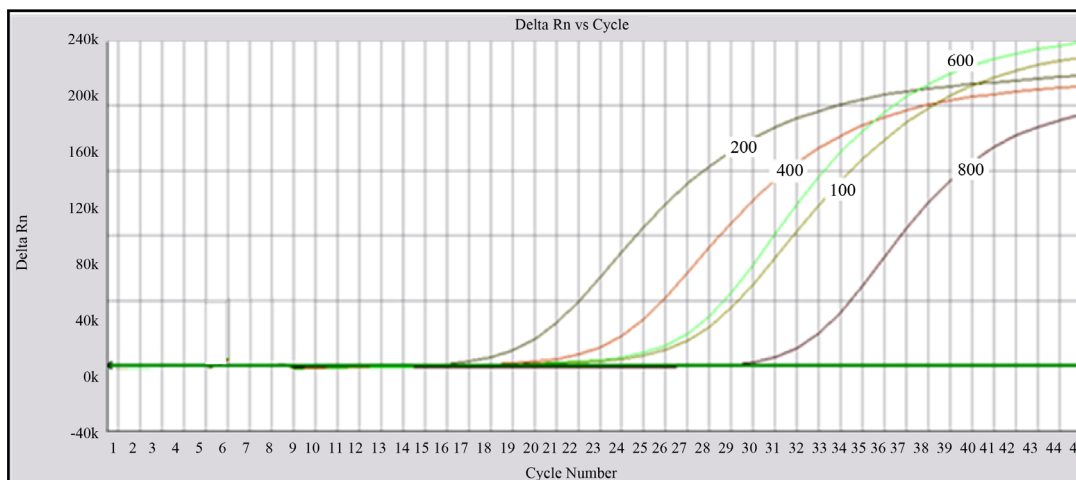

Figure 3. The best probe working concentration (*S. aureus*).

Figure 4. The best probe working concentration (*P. aeruginosa*).

extraction ranging from 105 to 10 copies DNA/μL, and the real-time PCR detection was carried out to establish standard curve. The assay sensitivity using an in vitro DNA standard for both duplex real-time and conventional RT-PCR assays were 50 copies/reaction. The result showed that the system was sensitive (**Figure 5**, **Figure 6**). Four duplicable tests showed that the coefficient of variation (CV) was less than 1%, which suggested that results of the developed duplex real-time PCR assay were very reproducible. Cell standard curves were constructed in duplicate for both species where a linear relationship was obtained between Ct and log CFU. The square regression coefficients showed a good correlation between the number of CFU and the Ct value. *P. aeruginosa* and *S. aureus* experiments variability data obtained were considered acceptable and were summarized in **Figure 7**, **Figure 8**.

3.3. Assay Specificity

This assay utilizes distinct gene sequences for *S. aureus* and *P. aeruginosa* in order to speciate strains referred for identification and typing. The assay showed positive results with Ct values of between 25 and 35 for either the *fem B* gene or *DNA gyrase subunit B* gene for isolates of *S. aureus* and *P. aeruginosa* respectively. There was no amplification with any of the unrelated microorganism isolates including *S. epidermidis*, *Shigella flexneri*, *L. monocytogenes*, *V. parahaemolyticus*, *E. coli O*157, *Shigella sonnei*, *E. faecalis*, *A. hydrophila/caviae*, *Y. enterocolitica*, *B. cereus*, *B. subtilis*, *E. coli*, *Salmonella*, *S. sanguinis*, *L. ivanovii*, *L. innocua* and 4 environmental isolates (**Figure 9**).

4. Discussion

The limitations of phenotypic methodologies including biochemical tests are previously documented, for example, serological assays used for the detection of *S. aureus* and *P. aeruginosa* often lack standardization and clinical sensitivity. A diagnostic alternative is the detection of *S. aureus* and *P. aeruginosa* antigen directly in clinical samples by means of an enzyme immunoassay or an immunofluorescent-antibody test. However, these methods lack sensitivity compared with nucleic acid amplification tests. A real-time PCR platform is preferable to conventional PCR for decreasing the risk of contamination and further reducing the total turnaround time.

The majority of isolates speciated by the reference laboratory are received as pure culture on charcoal transport swabs. The usual procedure requires culture as described, however in the case of urgent samples requiring a rapid result, direct DNA extraction from the charcoal transport medium has been applied in conjunction with this Taqman assay. With the use of the Roche MagNApure for the DNA extraction approximately 50 isolates have been successfully speciated with this method. Work in this area is ongoing with the applicability of the Taqman assay being tested with other non-culture samples including food and clinical specimens.

5. Conclusion

In conclusion, this assay has provided a rapid and reliable direct identification assay for *S. aureus* and *P.*
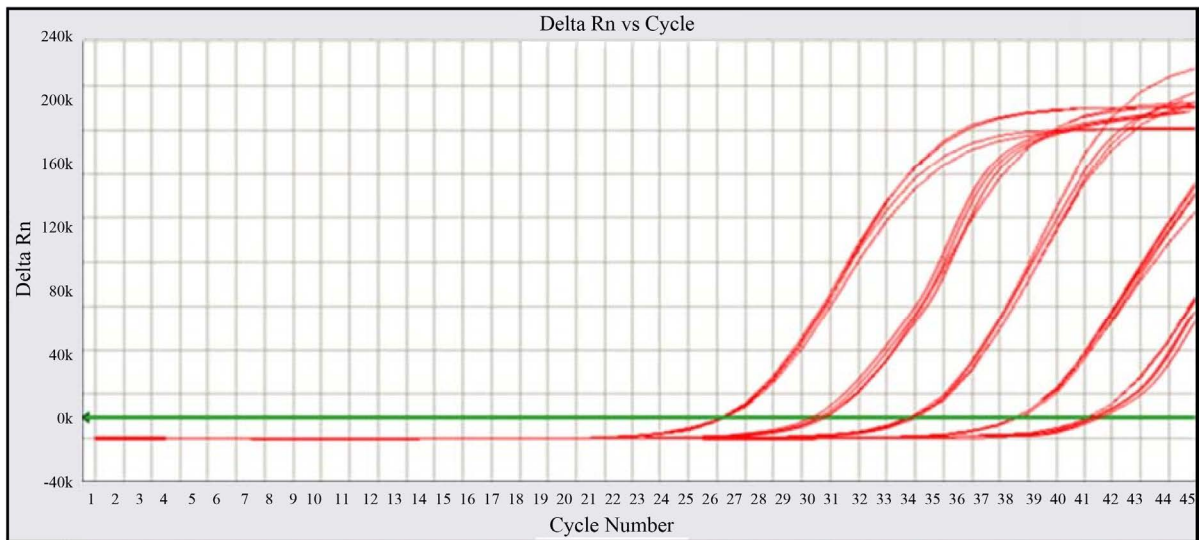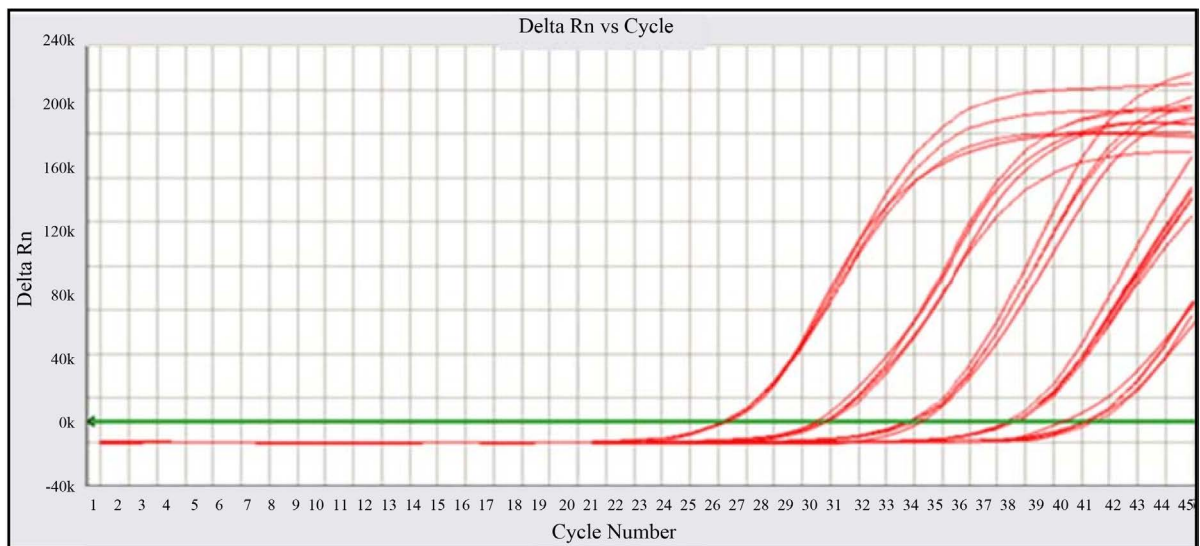
Figure 5. Sensitivity curves of *S. aureus*.

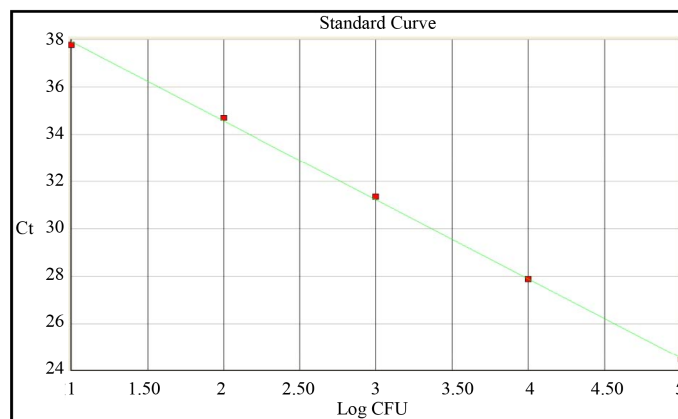
Figure 6. Sensitivity curves of *P. aeruginosa*.

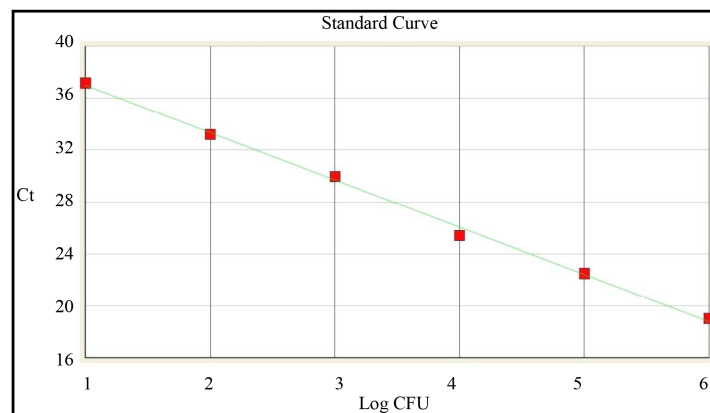**Figure 7.** Standard curve of *S. aureus*.

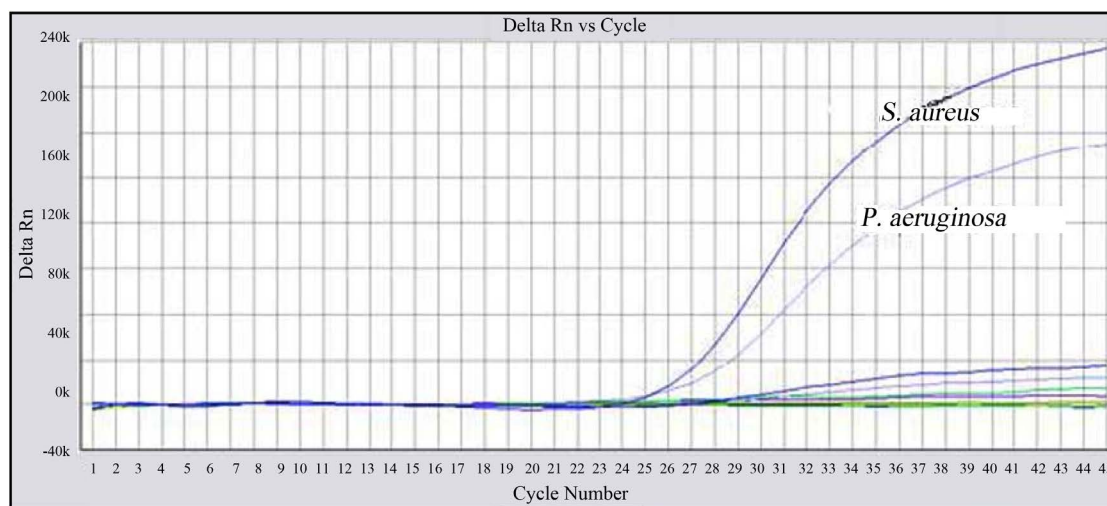**Figure 8.** Standard curve of *P. aeruginosa.*

Figure 9. The positive result of specific detection of *S. aureus* and *P. aeruginosa.*

aeruginosa, which can be applied to crude cell lysates of pure cultures, which is now being used as a primary speciation tool in this laboratory. Major advantages of the Taqman system include comparatively low reagent costs and the potential of integrating the process into automated data capture for laboratory information systems. Application to environmental samples and food samples is being evaluated. The use of the MagN Apure for rapid DNA extraction directly from transport swabs or samples linked to real-time PCR makes *S. aureus* and *P. aeruginosa* speciation possible within a single working day.

References

[1] Lowy, F.D. (1998) *Staphylococcus aureus* Infections. *New England Journal of Medicine*, **339**, 520-532. http://dx.doi.org/10.1056/NEJM199808203390806

[2] Hoiby, N., Frederiksen, B. and Pressler, T. (2005) Eradication of Early *Pseudomonas aeruginosa* Infection. *Journal of Cystic Fibrosis*, **4**, 49-54. http://www.sciencedirect.com/science/article/pii/S1569199305000688 http://dx.doi.org/10.1016/j.jcf.2005.05.018

[3] Kiska, D.L. and Gilligan, P.H. (2003) Pseudomonas. In: Murray, P.R., Baron, E.J., Jorgensen, J.H., Pfaller, M.A. and Yolken, R.H., Eds., *Manual of Clinical Microbiology*, 8th Edition, American Society for Microbiology Press, Washington DC, 719-728.

[4] Nogva, H.K., Bergh, A., Holck, A. and Rudi, K. (2000) Application of the 5' Nuclease PCR Assay in Evaluation and Development of Methods for Quantitative Detection of *Campylobacter jejuni*. *Applied and Environmental Microbiology*, **66**, 4029-4036. http://dx.doi.org/10.1128/AEM.66.9.4029-4036.2000

[5] Logan, J.M., Edwards, K.J., Saunders, N.A. and Stanley, J. (2001) Rapid Identification of *Campylobacter* spp. by

Melting Peak Analysis of Biprobes in Real-Time PCR. *Journal of Clinical Microbiology*, **39**, 2227-2232.
http://dx.doi.org/10.1128/JCM.39.6.2227-2232.2001

[6] Nogva, H.K., Naterstad, K.R.K., Holck, A. and Lillhaug, D. (2000) Application of 5'-Nuclease PCR for Quantitative Detection of *Listeria monocytogenes* in Pure Cultures, Water, Skim Milk and Unpasteurised Whole Milk. *Applied and Environmental Microbiology*, **66**, 4266-4271. http://dx.doi.org/10.1128/AEM.66.10.4266-4271.2000

[7] Chen, S., Yee, A., Griffiths, M., Larkin, C., Yamahiro, C.T., Behari, R., Paszko-Kolva, C., *et al*. (1997) The Evaluation of a Fluorogenic Polymerase Chain Reaction Assay for the Detection of *Salmonella* Species in Food Commodities. *International Journal of Food Microbiology*, **35**, 239-250. http://dx.doi.org/10.1016/S0168-1605(97)01241-5

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