

Primary research

Quantitation of *Pseudomonas aeruginosa* in wound biopsy samples: from bacterial culture to rapid 'real-time' polymerase chain reaction

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Statement of findings

We developed a real-time detection (RTD) polymerase chain reaction (PCR) with rapid thermal cycling to detect and quantify *Pseudomonas aeruginosa* in wound biopsy samples. This method produced a linear quantitative detection range of 7 logs, with a lower detection limit of 10³ colony-forming units (CFU)/g tissue or a few copies per reaction. The time from sample collection to result was less than 1 h. RTD-PCR has potential for rapid quantitative detection of pathogens in critical care patients, enabling early and individualized treatment.

Keywords: burn wound, polymerase chain reaction, *Pseudomonas aeruginosa*, quantitation, sepsis

Synopsis

Introduction: Early diagnosis of wound colonisation or prediction of wound sepsis provides an opportunity for therapeutic intervention. There is need for qualitative and quantitative tests that are more rapid than bacterial culture. *Pseudomonas aeruginosa* results in high morbidity and mortality rates, is inherently resistant to common antibiotics, and is increasingly being isolated as a nosocomial pathogen. We developed three PCR-based methods to detect and quantify *P aeruginosa* in wound biopsy samples: conventional PCR, enzyme-linked immunosorbent assay (ELISA)-PCR, and RTD-PCR with rapid thermal cycling (LightCycler™ technology), all based on the amplification of the outer membrane lipoprotein gene *oprL*. We compared the efficacy of these methods to bacterial culture by quantitatively measuring levels of *P aeruginosa* in serial dilutions, in reconstituted skin samples and 21 burn wound biopsy samples.

Materials and methods: Serial 10-fold dilutions were made from an overnight *P aeruginosa* culture and plated out onto Luria-Bertani and cetrinide agar plates. The agar plates were incubated overnight at 37°C, and the colonies were counted in order to estimate the number of CFU per dilution tube. A

sample was taken from each dilution tube as a template for the three PCR-based methods.

Serial *P aeruginosa* dilutions (see above) were added to uninfected cadaveric skin. The reconstituted biopsy samples were homogenized using a tissue tearer and DNA was extracted using XTRAX DNA buffer. The DNA was resuspended in distilled water. A sample was taken as a template for the PCR-based methods.

Twenty-one burn wound biopsy samples were taken from nine patients with suspected *P aeruginosa* burn wound infection. The biopsy samples were longitudinally divided into two pieces. From one piece, DNA was extracted (using XTRAX DNA buffer) and used as a template for PCR-based techniques (see above). The other piece was homogenized, in physiological water, using a tissue tearer. Serial 10-fold dilutions of the suspension were spread on Luria-Bertani and cetrinide agar plates. Colony counts were performed after overnight incubation at 37°C.

The PCR mixture contained sterile distilled water, PCR buffer, deoxynucleotide mixture or digoxigenin labelling mix, MgCl₂, diluted template, primers PAL1 and PAL2, and AmpliTaq DNA polymerase. The amplification was performed in a GeneAmp®

CFU = colony-forming units; ELISA = enzyme-linked immunosorbent assay; EtBr = ethidium bromide; MTP = microtitre plate; PCR = polymerase chain reaction; RTD = real-time detection.

PCR System 2400. An aliquot of the reaction mixture was put on an agarose gel for electrophoresis and visualisation of the PCR product. An image of the gel was made using a digital camera. Image analysis software was used to calculate the band mass of the experimental bands.

An aliquot of the digoxigenin labelling reaction was denatured and then hybridized with the biotinylated capture probe PrL. Some of the resultant solution was transferred to a well of a streptavidin-coated microtitre plate (MTP) and incubated for 3 h at 45°C. The solution was discarded. Peroxidase conjugated antidigoxigenin was added and the MTP was incubated for 30 min at 37°C. The solution was discarded and ABTS substrate was added. The MTP was incubated for 30 min at 37°C. Absorbance was read at 405 nm.

The RTD-PCR mixture contained PCR grade sterile water, diluted template DNA, primers PAL1 and PAL2, 3' fluorescein (FL)-labelled probe oprL-FL, 5' LC Red 640-labelled and 3' phosphorylated probe oprL-LC, MgCl₂, and LC DNA Master Hybridisation Probes, containing *Taq* DNA polymerase, reaction buffer, dNTP mix with dUTP instead of dTTP and, MgCl₂. The amplification was performed in a LightCycler™. The fluorescence signal of LC Red 640 was measured during the

annealing phase. The measured fluorescence data was processed with analysis software.

Results and discussion: The three methods showed a good concordance with the culture results. Conventional PCR was at least 100 times less sensitive than bacterial culture and had a low dynamic range (2 logs). With a lower detection limit of 10³ CFU/g tissue, ELISA-PCR was ten times more sensitive than conventional PCR. The dynamic range, however, did not increase. ELISA-PCR is very time consuming (8 h). The RTD-PCR produced a linear quantitative detection range of 7 logs with a lower detection limit of 10³ CFU/g tissue. More important, however, was that the time from sample collection to result was less than 1 h. Two biopsy specimen scored significantly higher in ELISA-PCR and RTD-PCR than in bacterial culture. This could indicate that DNA from dead bacteria was amplified. One out of ten culture positive biopsy samples was found negative by all PCR-based methods. Topical antimicrobial agents possibly inhibited PCR. These results show that RTD-PCR has potential for the rapid quantitative detection of pathogens in critical care patients, enabling early and individualized treatment. Further study is required to assess the reliability of this new technology, and its impact on patient outcome and hospital costs.

Full article

Introduction

Although effective topical antimicrobial chemotherapy and early excision of burn wounds have significantly reduced the occurrence of invasive burn wound infections, sepsis is still a major problem [1–3]. The risk of septicaemia increases in proportion to the degree of cutaneous infection [4–6]. Many investigators have reported [6–11] that quantitative biopsy culture was the best method for early detection of sepsis. Heininger *et al* [12] stressed that only 4–12% of blood cultures is found positive. Conversely, McManus *et al* [13] reported in 1987 that high tissue counts did not necessarily indicate invasion, and that the principal value of quantitative biopsy culture was the demonstration of the predominant burn wound flora. Even so, when sepsis ensues, while awaiting the results of blood cultures, a knowledge of the organisms that colonize a burn wound can facilitate prompt and appropriate antibiotic treatment that is based on the expected sensitivity of the identified germs, rather than initiating a purely empirical therapy. There is need for qualitative and quantitative tests that are more rapid than bacterial culture.

We decided to develop such a test for the rapid detection and quantitation of *Pseudomonas aeruginosa* in burn wound biopsy samples. This bacterium is ubiquitous, is inherently resistant to common antibiotics, and therefore is one of the most problematic pathogens in modern hospitals

[14–17]. Burn wound patients, mechanically ventilated patients and cystic fibrosis patients are particularly susceptible to *P aeruginosa* infection [1,18,19]. Biopsy samples are, with respect to homogenization and DNA extraction, very tough clinical samples and thus would be an excellent test case for the applicability of the method on direct clinical samples.

In 1997 our group developed a PCR test for the direct detection and identification of *P aeruginosa* in clinical samples that is based on the amplification of the outer membrane lipoprotein gene *oprL* [20–22]. Since then we developed several quantitative variants of this test, exploiting the technology available at the time.

In the first instance, we amplified the *oprL* gene by means of conventional PCR and visualized the PCR product by ethidium bromide (EtBr) staining of agarose gels. The intensity of the fluorescence produced by EtBr was quantified.

Second, we developed an ELISA-mediated PCR in order to quantify the amplified *oprL* gene. PCR products were labelled with digoxigenin during the amplification process and quantitatively detected by absorbance reading in microtitre plates.

Finally, we exploited the recently developed 'real-time' quantitative PCR technology [23–27]. We opted for the

LightCycler™ [28] system (Roche Diagnostics, Brussels, Belgium) because it features rapid capillary tube resistive thermal cycling, reducing the amplification time dramatically. Two adjacent hybridization probes, labelled with different fluorescent dyes, are used to monitor the appearance of PCR product. The emitted light signal is proportional to the amount of specific DNA product available for hybridization, and thus increases every cycle [29]. The probes were designed to be complementary to a conserved region of the *oprL* gene, as determined by sequence analysis of the *oprL* gene of 85 nonrelated clinical *P aeruginosa* isolates.

In the present report we compare the assay performance of the above-mentioned methods, in terms of practicability, to bacterial culture. For this purpose three types of samples were assayed: serial *P aeruginosa* dilutions, uninfected skin spiked with *P aeruginosa* and 21 burn wound biopsy samples. All methods were useful, but only LightCycler™ RTD-PCR allowed rapid quantitative detection of *P aeruginosa* in skin biopsies with an adequate detection limit and a wide log-linear range.

Materials and methods

Sample preparation

Serial Pseudomonas aeruginosa dilutions

Serial 10-fold dilutions were made from an overnight *P aeruginosa* culture (strain PAO-1, ATCC 15692) in order to look for the lower detection limit and the dynamic range of the PCR-based methods. Dilutions were made in physiological water. Aliquots (100 µl) were plated out, in triplicate, onto Luria-Bertani (Gibco-BRL Life Technologies, Paisley, Scotland) and cetrinide (Sanofi Pasteur, Brussels, Belgium) agar plates. The agar plates were incubated overnight at 37°C, and the colonies were counted in order to estimate the number of CFU per dilution tube. A sample (5 µl) was taken from each dilution tube as a template for the three PCR-based methods. Standards ranging from 1 to 10⁶ CFU/5 µl were assayed.

Reconstituted biopsy samples

Reconstituted biopsy samples were prepared in sterile 5-ml tubes (Nunc; Roskilde, Denmark) by adding 10 µl serial *P aeruginosa* dilutions (see above) to 50 mg uninfected cadaveric skin, at final concentrations of 10–10⁹ CFU/g tissue. XTRAX DNA extraction buffer (1 ml; Gull Laboratories, Salt Lake City, UT, USA) was added, and the reconstituted biopsy samples were homogenized for 1 min, at 30 000 rpm, using a tissue tearer (BioSpec Products, Bartlesville, Oklahoma, USA). The suspensions were transferred to a 1.8-ml cryotube (Nunc) with a loosened cap and were microwaved for 13 s at 600 W. The tubes were gently shaken to mix the contents and were microwaved for a further 6 s. The tubes were cooled for 3 min at room temperature and centrifuged for 1 min at 10 000 g in order to pellet the proteins.

Supernatant (500 µl) was transferred to a microcentrifuge tube (Eppendorf, Hamburg, Germany). Molecular grade isopropanol (500 µl; Sigma Aldrich, Deisenhofen, Germany) was added, and the contents of the tube were mixed thoroughly by inverting the tube 10 times. The tube was centrifuged for 1 min at 10 000 g in order to pellet the DNA. The supernatant was discarded by decanting, and the tube was allowed to stand upside down for 2 min to allow the remaining fluid to drain. The DNA was resuspended in 30 µl distilled water. A sample (5 µl) was taken as a template for conventional PCR and ELISA-PCR, and 3 µl was taken for RTD-PCR. Standards ranging from 10³ to 10⁹ CFU/g skin tissue were assayed.

Clinical burn wound biopsy samples

Twenty-one burn wound biopsy samples, weighing 20–170 mg (mean 54 mg), were assayed. The biopsy specimens were aseptically taken from nine patients with suspected *P aeruginosa* burn wound infection, using a 4-mm punch biopsy needle (Labo Stiefel, Leuven, Belgium; *n* = 15) or a lancet (*n* = 6).

The samples were longitudinally divided into two pieces using a sterile scalpel and weighed. From one piece DNA was extracted using XTRAX DNA buffer and used as template for the PCR-based techniques (see above). The other piece was collected in a sterile 5-ml tube containing 10 µl physiological water/mg tissue, and was homogenized, on ice, for 1 min at 30 000 rpm using a tissue tearer. Serial 10-fold dilutions of the homogenized wound biopsy samples were spread, in triplicate, on Luria-Bertani and cetrinide agar plates. Colony counts were performed after overnight incubation at 37°C.

Conventional polymerase chain reaction and digoxigenin labelling

The PCR was completed in 200-µl microcentrifuge tubes. The PCR mixture (50 µl final volume) contained the following: 26.5 µl sterile distilled water; 5 µl 10 × PCR buffer (500 mmol/l KCl and 100 mmol/l Tris-HCl; pH 8.3); 5 µl of a deoxynucleotide mixture (dGTP, dTTP, dATP and dCTP; 2 mmol/l each) or 5 µl digoxigenin-labelling mix from Boehringer-Mannheim (Brussels, Belgium; 2 mmol/l dGTP, dATP and dCTP, and 1.9 mmol/l digoxigenin-dUTP); 6 µl MgCl₂ (2.5 mmol/l); 5 µl diluted template DNA; 1 µl primer PAL1 (25 µmol/l); 1 µl primer PAL2 (25 µmol/l); and 0.5 µl AmpliTaq DNA polymerase (5 U/µl). The primers had the following sequences: PAL1, 5'-ATGGAAATGCTGAAATTCGGC-3'; and PAL2, 5'-CTTCTTCAGCTCGACGCGACG-3'. All PCR reagents were purchased from PE Applied Biosystems (Nieuwerkerk a/d IJssel, The Netherlands).

A reaction mixture containing all of the ingredients except the template was made. The amplification was performed in a GeneAmp® PCR System 2400 (PE Applied Biosystems).

The amplification program was set at 35 cycles of denaturation at 94°C for 30 s or 45 s (digoxigenin labelling), annealing at 57°C for 30 s or 1 min (digoxigenin labelling), and elongation at 72°C for 30 s or 2 min (digoxigenin labelling).

Some of the reaction mixture (10 µl) was put on an agarose gel of 2% (weight/volume) for electrophoresis and visualization of the PCR product after staining with EtBr on a ultraviolet transilluminator. An image of the gel was made using a DC40 digital camera (Eastman Kodak Company, Rochester, New York, USA). Using the intensity measured for the bands originating from the standards, *Paeruginosa* serial dilutions or reconstituted samples, Kodak Digital Science 1D image analysis software was used to calculate the band mass of the experimental bands using linear regression.

Digoxigenin detection

Some of the digoxigenin labelling reaction (35 µl) was transferred to a sterile microcentrifuge tube (Eppendorf). Denaturation solution (40 µl) was added and the tube was incubated for 10 min at room temperature. The tube was filled up to 500 µl with hybridization solution, which contained 50 pmol/ml biotinylated capture probe PrL with the following sequence: 5'-AAGCCGGAAGCCATGCGCGCT-3'. Some of the resultant solution (200 µl) was transferred to a well of a streptavidin-coated microtitre plate (MTP). The MTP was incubated for 3 h at 45°C on a MTP shaker. The solution was discarded and the well was washed five times with 250 µl washing solution. A quantity (200 µl) of a peroxidase conjugated antidigoxigenin solution (10 mU/ml) was added, and the MTP was incubated for 30 min at 37°C on a MTP shaker. The solution was discarded and the well was washed five times with washing solution. ABTS substrate solution (200 µl) was added and the MTP was incubated for 30 min at 37°C on a MTP shaker. Absorbance was read at 405 nm. All digoxigenin detection reagents were purchased from Boehringer-Mannheim.

Real-time detection polymerase chain reaction

The RTD-PCR mixture (20 µl final volume) contained the following: 3.8 µl PCR grade sterile water; 3 µl diluted template DNA; 2 µl primer PAL1 (5 µmol/l); 2 µl primer PAL2 (5 µmol/l); 2 µl of the 3' fluorescein-labelled probe *oprL*-FL (2 µmol/l); 4 µl of 5' LC Red 640-labelled and 3' phosphorylated probe *oprL*-LC (2 µmol/l); 1.2 µl MgCl₂ (25 mmol/l); 2 µl LC DNA Master Hybridisation Probes containing *Taq* DNA polymerase; reaction buffer; dNTP mix with dUTP instead of dTTP; and 10 mmol/l MgCl₂. All reagents were purchased from Roche Molecular Biochemicals (Brussels, Belgium). The probes were manufactured by the TIB MOLBIOL synthesis laboratory in Berlin, Germany, and had the following sequences: *oprL*-FL, 5'-TGCGATCACCACTTCTACTTCGAGT-FL-3'; and *oprL*-LC, 5'-LC Red 640-CGACAGCTCCGACCTGAAG-p-3'.

Samples were spun into glass capillary tubes, capped and placed in the LightCycler™. After an initial denaturation at 95°C for 30 s, amplification was performed for 45 cycles of denaturation at 95°C for 2 s, annealing at 59°C for 10 s and elongation at 72°C for 10 s. The fluorescence signal of LC Red 640 was measured during the annealing phase. The measured fluorescence data was processed with analysis software.

Results and discussion

Conventional polymerase chain reaction

With a lower detection level between 10⁴ and 10⁵ CFU/g, wound biopsy tissue conventional PCR was at least 100 times less sensitive than bacterial culture (Table 1). This problem was related to the limited amount of sample, as compared with bacterial culture, that can be incorporated into one PCR reaction. Concentration of DNA or division of the sample over multiple PCR reactions could resolve this problem, but would result in a more elaborate method. Conventional PCR had a low dynamic range; bands that were very close to the background level gave poor results, and bands that were saturated also skewed the results (Fig. 1).

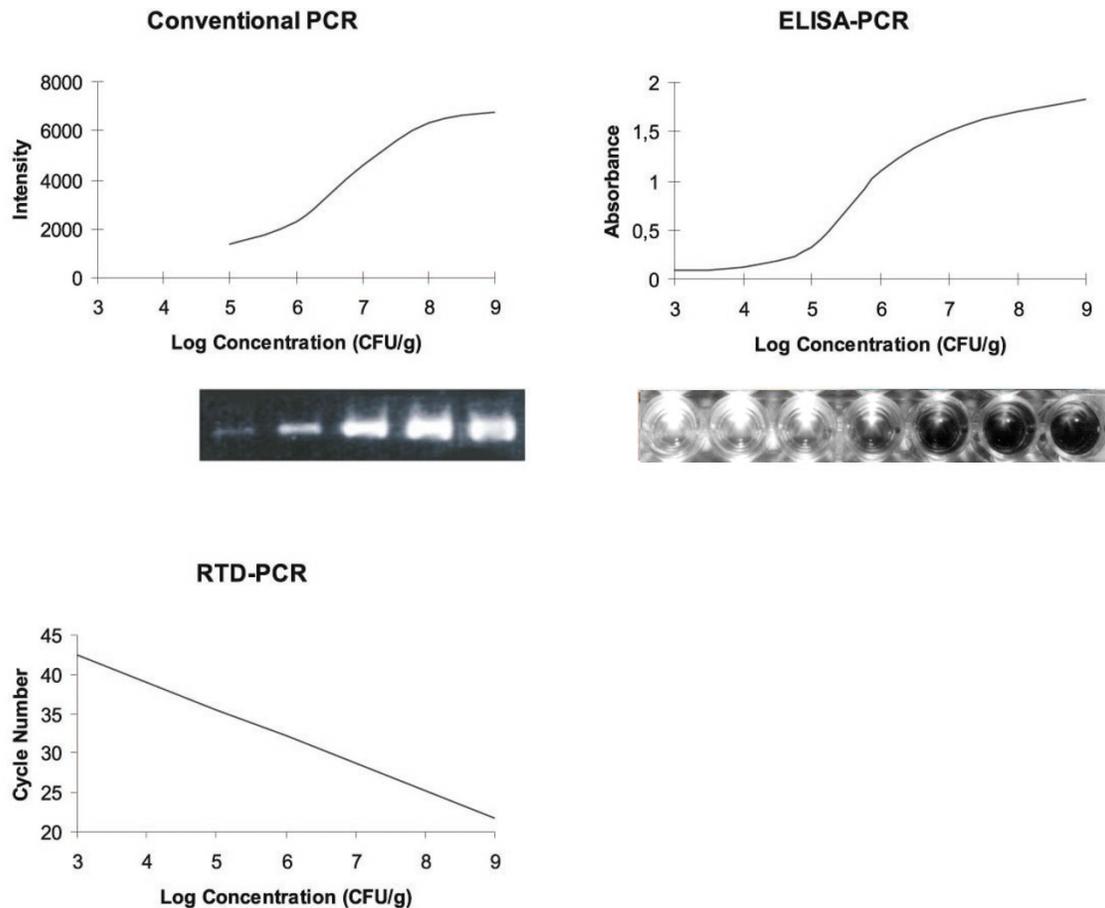
Ten out of 21 burn wound biopsy samples turned out to be culture positive; five of them, with a density lower than 10⁴ CFU/g, were not detected by conventional PCR. All culture-negative samples were also negative in PCR.

Enzyme-linked immunosorbent assay polymerase chain reaction

With a detection limit of 10³ CFU/g tissue, ELISA-PCR was at least 10 times more sensitive than conventional PCR (Table 1). However, the linear response range did not increase, but shifted to lower concentrations along with the detection limit (Fig. 1). This technique is very time consuming and involves handling that increase the risk of contamination of PCR product (Fig. 2). Only one culture-positive wound biopsy, out of 10, containing 3000 CFU/g tissue, was found to be negative by ELISA-PCR. Topical antimicrobial agents possibly inhibited PCR.

Two biopsy specimens with approximately 200 and 400 CFU/g tissue, as determined by bacterial culture, scored significantly higher in ELISA-PCR: 1200 and 1450 CFU/g tissue, respectively. This could indicate that DNA from nonproliferating or dead bacteria was amplified. The question regarding whether quantitative estimates of bacteria, determined using PCR-based methods, correlate with the number of viable bacteria has been raised before [30]. For this reason, PCR-based tests are probably not appropriate for the monitoring of treatment efficacy. They should be considered as complementary indicative tests in critical care settings. On the other hand, PCR-based methods could detect bacteria that were inhibited in their growth by residual concentrations of antimicrobials in the sample [31], overgrown by other bacteria present in the

Figure 1



Standard curves for reconstituted biopsy samples.

sample, autolyzed during incubation [32], or difficult to cultivate *in vitro* [33], and are thus under-diagnosed by bacterial culture. PCR-ELISA has merit with regard to its sensitivity, but is too time-consuming and expensive to implement in a clinical laboratory (Fig. 2).

Real-time detection polymerase chain reaction

RTD-PCR produced a linear quantitative detection range of 7 logs with a lower detection limit of 10^3 CFU/g tissue, or a few copies per reaction (Table 1). RTD-PCR uses a kinetic approach rather than an end-point approach. Kinetic quantification in the log phase of the reaction, the phase of constant efficiency, clearly generates a larger linear response than end-point detection in the plateau phase of the reaction (Fig. 1). Also, there is no need for any post-PCR sample manipulation, eliminating PCR contamination concerns. The recent introduction of a second dye for the LightCycler™ system offers the prospect of an internal control, addressing the problem of tube-to-tube variations and PCR inhibition, and thus improving the reliability of the results. However, the greatest merit of Light-

Cycler™ RTD-PCR is its rapidity. Rapid thermal cycling reduced the time of amplification, detection and analysis of DNA from several hours to 30 min (Fig. 2). The RTD-PCR results for the burn wound biopsy samples were concordant with those obtained using ELISA-PCR; the false-negative result and the two over-diagnosed biopsies were also observed using RTD-PCR. The use of capillaries instead of tubes permits smaller reaction volumes, thus lowering the reagent costs. The set-up costs, however, may be beyond the capabilities of some laboratories (Table 1).

Conclusion

Up until now, RTD-PCR has been applied for the detection of food-borne pathogens [34], cancer [35–37], genetic diseases [38] and infectious diseases [39–42].

Although a limited number of clinical specimens were tested, the present results indicate that RTD-PCR, and more specifically LightCycler™ technology, has potential for quantitative applications in the clinical laboratory. In

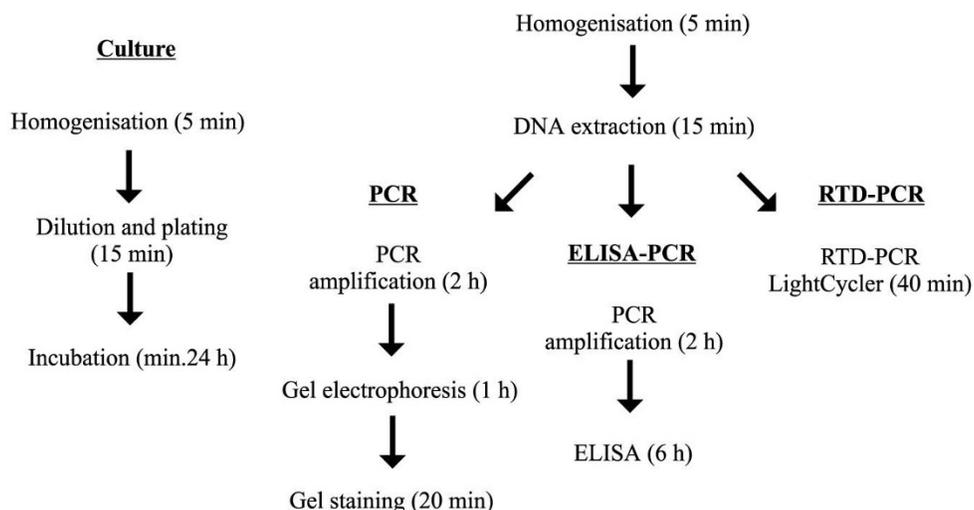
Table 1

Summary of the characteristics of the tested quantitation methods

Method	Lower detection limit*		Log-linear range (CFU/g)	Time to result (h)	Ease of use	Cost per test (US\$) [†]	Set up cost (\$)
	(CFU/g)	(CFU/reaction)					
Culture	10 ²	1	10 ² –10 ⁹	24	Easy	6	3500
PCR	10 ⁴ –10 ⁵	10–10 ²	10 ⁶ –10 ⁸	4	Moderate	12 [‡]	20 000
ELISA-PCR	10 ³ –10 ⁴	1–10	10 ⁵ –10 ⁷	8	Elaborate	25 [‡]	20 000
RTD-PCR (LC)	10 ³ –10 ⁴	1–10	10 ³ –10 ⁹	1	Moderate	9 [‡]	73 000

*The lower detection limit is variable due to variations in inter-run amplification efficiency. [†]Not including labour costs, only reagents and disposables. [‡]Cost per clinical sample, based on the analysis of batches of 10 clinical samples and eight standards. LC, LightCycler™.

Figure 2



Comparison of the procedural steps involved in the quantitation methods.

particular, it has applications for the critical care population, at the point of care, and it is important that the test is subjected to further optimization and evaluation. Early infection diagnosis remains a difficult problem for patients with burn wounds or cystic fibrosis, and for critical care patients in general. Prognosis and survival are often dependent on an early, individualized treatment. Automated extraction of DNA from a variety of clinical samples (blood, expectorations, urine, etc.) and subsequent rapid, online, quantitative detection of pathogens (*P aeruginosa*, *Staphylococcus aureus*, *Haemophilus influenzae*, among others) by RTD-PCR is now possible, allowing early therapeutic decisions to be made. Multiple colour detection will open the door to multiplex RTD-PCR. Further studies are necessary to assess the impact of rapid RTD-PCR on patient outcome and hospital costs [43,44].

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