# CHAPTER 5: RAPID DETECTION AND SEQUENCE-SPECIFIC DIFFERENTIATION OF EXTENDED-SPECTRUM BETA-LACTAMASE GES-2 FROM *PSEUDOMONAS AERUGINOSA*, WITH A REAL-TIME PCR ASSAY.

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## 5.1 Introduction

Detection of GES-2 and other extended-spectrum beta-lactamase (ESBL)-producing *Pseudomonas aeruginosa* isolates in the clinical microbiology laboratory utilizing ordinary methods is notoriously difficult and laborious (20). In addition, the integron genetic structures that support these ESBLs such as GES-2, not only confers resistance towards broad-spectrum beta-lactam antibiotics, but also towards other unrelated classes of antibiotics such as aminoglycosides as well as several non-related chemical compounds (20). These isolates are therefore very difficult indeed, to treat and control successfully. Apart from standard PCR and gene sequencing, molecular detection methods for ESBLs in general developed a wide array of applications most suited for the TEM- and SHV-type of ESBL families (4). These methods may include oligotyping (10), PCR-restriction fragment length polymorphism analysis (2), single-strand conformation polymorphism analysis (12), real-time PCR with melting curve analysis (18), and ligase chain reaction-based tests (7, 14). This chapter describes the application of the LightCycler to rapidly and specifically detect the more potent gene, $bla_{GES-2}$, in the GES / IBC ESBL family.

## 5.2 Materials and methods

**5.2.1 Bacterial strains.** One hundred *P. aeruginosa* clinical isolates, as described in Chapter 4, were included in this study (section 4.2.1). Well-characterized bacterial isolates used to standardize the molecular detection methods are listed in Table 4-1.

**5.2.2 Susceptibility testing.** A disk diffusion assay as described in Chapter 4 was performed and results interpreted (section 4.2.2, 13).

**5.2.3 DNA extraction.** Extraction of whole-cell DNA was accomplished with an ethanol precipitation-based method as described previously (section 4.2.3, 6,15).

**5.2.4 LightCycler fluorescence resonance energy transfer (FRET) mediated mutation assay.** The mutation detection assay was performed as described previously (18), with reaction specific modifications as necessary.

**i) Amplification primer design.** A forward primer GES-C, 5`-GTT TTG CAA TGT GCT CAA CG-3` (nt 176 – 195) (*Tm* = 54.6°C), was designed from the gene sequence of *bla*$_{GES-2}$ [GenBank accession number AF 326355] (17), by utilizing Primer3 software (Whitehead Institute for Biomedical Research). The previously described primer GES-1B (20-mer, *Tm* = 56.4°C) (17, 20), was used as the reverse primer, amplifying a 689 bp product. Both primers were synthesized and purified by Integrated DNA Technologies Inc., Coralville, IO.

**ii) Fluorogenic probe design.** Since the GG to AA mutation defining *bla*$_{GES-2}$ from *bla*$_{GES-1}$ and *bla*$_{IBC1/2}$ respectively, resides on nt 493 – 494 of the coding region (17), a 20-mer, 3`-FITC labeled sensor probe, 5`-AGA TGG GCG ACA ACA CAC CT-3` (*Tm* = 59.8°C), was designed from the gene sequence of *bla*$_{GES-1}$ [GenBank accession number AF 156486] (16). The anchor probe consisted of a 33-mer, 5`-LC Red640 labeled and 3`-phosphorylated, 5`- GCG ACC TCA GAG ATA CAA CTA CGC CTA TTG CTA-3` (*Tm* = 68.4°C) oligonucleotide, binding at a distance of one nucleotide, directly downstream of the sensor probe (Table 5-1).

**Table 5-1: Oligonucleotide sequences for PCR analysis used in this study.**

| Primer / probe | Sequence [a] | Function | Source or reference |
|---|---|---|---|
| **Nested PCR** | | | |
| GES-1A | ATG CGC TTC ATT CAC GCA C | Forward primer | 17, 20 |
| GES-1B | CTA TTT GTC CGT GCT CAG G | Reverse primer | 17, 20 |
| GES-C | GTT TTG CAA TGT GCT CAA CG | Forward primer | This study |
| GES-D | TGC CAT AGC AAT AGG CGT AG | Reverse primer | This study |
| **Real-time PCR** | | | |
| GES-C | GTT TTG CAA TGT GCT CAA CG | Forward primer | This study |
| GES-1B | CTA TTT GTC CGT GCT CAG G | Reverse primer | 17, 20 |
| Probe 1 | AGA TGG GCG ACA ACA CAC CT - FL | Sensor probe complementary to $bla_{GES-1}$ / $bla_{IBC}$ | This study |
| Probe 2 | LC Red 640- GCG ACC TCA GAG ATA CAA CTA CGC CTA TTG CTA - PH | Anchor probe | This study |

[a] – Sequences are delineated in 5` - 3` order. Shaded box depict relevant point mutation. FL – fluorescein, PH – phosphorylation, LC Red 640 – LightCycler Red 640.

**Table 5-2: LightCycler amplification and melting curve protocol followed in this study.**

| Program | Number of cycles | Target temperature (ºC) | Hold time (s) | Temperature transition rate (ºC/s) | Fluorescence acquisition mode [a] |
|---|---|---|---|---|---|
| **Polymerase activation** | 01 | 95 | 600 | 20 | None |
| **Three-step PCR** | 40 | | | | |
| a) Denaturation cycle | | 96 | 01 | 20 | None |
| b) Amplification cycle | | 52 | 10 | 20 | Single |
| c) Extension cycle | | 72 | 15 | 20 | None |
| **Three step melting curve** | 01 | | | | |
| a) Denaturation cycle | | 95 | 10 | 20 | None |
| b) Holding cycle | | 45 | 30 | 20 | None |
| c) Melting cycle | | 95 | 0 | 0.2 | Continuous |

[a] Fluorimeter gains settings were: F1 equal to 1, F2 equal to 15 and F3 equal to 30.

Both fluorophore–labeled probes were synthesized and purified by reversed-phase high-pressure liquid chromatography by TIB Mol Biol (Berlin, Germany). Primer and probe placement on the DNA template is depicted in Figure 5-1.

**iii) Preparation of PCR reaction.** The PCR reaction mix comprised 0.5 µM of each primer, 0.2 µM of the sensor and anchor probes respectively, 4 µl LightCycler FastStart DNA Master$^{PLUS}$ reaction mix (Roche Diagnostics, Penzberg, Germany), 5 µl DNA template and nuclease free water to a final reaction volume of 20 µl. This reaction mix was loaded into glass capillary cuvettes and after brief centrifugation (20 rpm for 30 s), the sealed capillaries were loaded into the LightCycler (Idaho Technology Inc., Salt Lake City, UT) for rapid PCR analysis. Controls included DNA templates from well-characterized isolates (Table 4-1) as well as distilled water as a DNA negative control.

**iv) LightCycler program and melting curve analysis.** Programming of the LightCycler (Table 5-2), consisted of four distinct steps namely: a) one cycle for polymerase activation and template denaturation (10 min at 95°C), b) 40 cycles each comprising of denaturation (one second (s) at 96°C), annealing (10 s at 52°C) with single fluorescence acquisition mode and extension (15 s at 72°C) with a temperature transition rate of 20°C/s, c) a melting curve comprising of one denaturation cycle (10 s at 95°C), one holding cycle (30 s at 45°C) and one melting cycle with a target temperature of 95°C (temperature transition rate 0.2°C/s) with continuous fluorescence acquisition mode, and finally d) one cooling cycle to 40°C (temperature transition rate 20°C/s). Optimum automated fluorimeter gains settings as determined by LightCycler Software version 3.5 (Idaho Technology Inc., Salt Lake City, UT) comprised of: F1 equal to 1, F2 equal to 15 and F3 equal to 30. Continuous fluorescence monitoring during the melting cycle measured the decrease of FRET at

fluorescence F2, as the FITC-labeled sensor probe dissociated from the LC Red640-labelled anchor probe during the slow temperature rise. Fluorescence signals from F2 were plotted automatically in real-time against temperature (*T*) to produce a melting curve for the mutation occurring at nt 493 - 494. Automated conversion of this data to melting peaks was obtained by plotting the negative derivative of fluorescence (-*dF2/dT*) versus *T*.

**v) Analysis of amplification products.** The entire contents of capillaries containing amplification products from well-characterized isolates (Table 4-1), were analyzed by means of agarose gel electrophoresis as previously described (18).

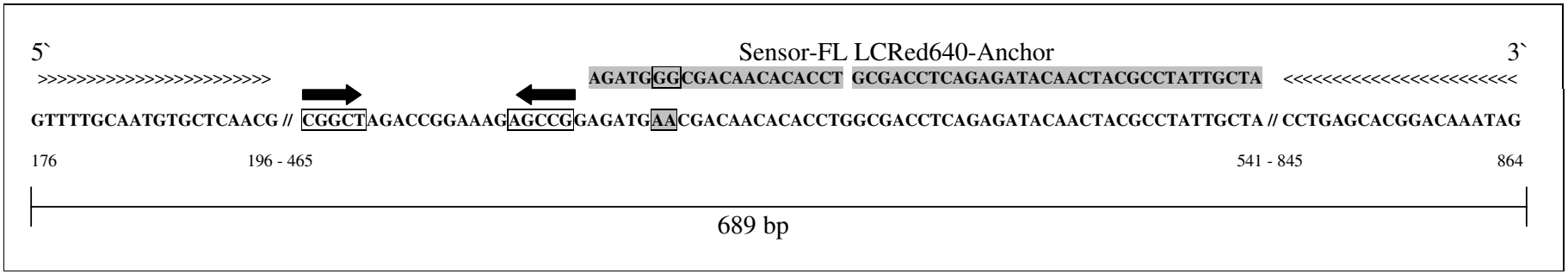


**Figure 5-1:** Alignment of real-time PCR primer and fluorogenic probe sequences with *bla*$_{GES-2}$. Stem loop sequences appear in boxes, arrows indicate the direction of stem loop formation. The relevant nucleotide mismatch (nt 493 – 494) is depicted as a shaded box. Note the close approximation of the sensor probe to the stem loop structure in addition to the one nt distance between the sensor and anchor probes respectively. [>>> Sense and <<< Antisense primers] [nt 488 – 507 sensor probe 3` marked with fluorescein (FL) and nt 509 – 541 anchor probe 5` marked with LightCycler Red 640 (LCR640)].

**5.2.5 Nested-PCR amplification.**

**i) Nested-PCR primer design.** Previously published primers GES-1A and GES-1B (17, 20), were used to amplify the entire *bla*$_{GES\ /\ IBC}$ coding region during the first round of amplification. For a second round of amplification, an alternative reverse primer was constructed from the gene sequence of *bla*$_{GES-2}$ [GenBank accession number AF 326355] (17), utilizing Primer3 software (Whitehead Institute). The following primer sequence was generated: GES-D, 5`-TGC CAT AGC AAT AGG CGT AG-3` (nt 527 – 546), targeting a 371 bp region in conjunction with forward primer GES-C (Table 5-1), spanning all currently published point mutations of *bla*$_{GES}$ and *bla*$_{IBC}$ genes (5, 11, 16, 17).

**ii) Nested PCR reaction conditions.** Primers GES-1A and GES-1B were first utilized under standard PCR conditions (19), to amplify an 864 bp product. The first round PCR reaction mix comprised 0.32 µM of each primer, 5 µl *Taq* DNA reaction buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 1.25 U *Taq* DNA polymerase, (Promega Corporation, Madison, WI), 5 µl DNA template and distilled water to a final reaction volume of 50 µl. Amplification was performed with a GeneAmp PCR System 9600 (Perkin Elmer Cetus, Emeryville, CA) and consisted of an initial denaturation step (2 min at 95°C), followed by 35 amplification cycles each comprising a denaturation step (30 s at 95°C), followed by annealing (1 min at 50°C), and extension (1 min at 72°C). Completion of 35 amplification cycles was finally followed by an extension step of 5 min at 72°C. Second round amplification conditions were exactly similar to the first round, primers GES-C and GES-D were used together with 2 µl of DNA product obtained from first round amplification as a reaction template. All reaction preparations were performed in separate rooms

equipped with laminar flow cabinets to avoid cross-contamination with amplification products. PCR products obtained from both amplification rounds were electrophoresed as described previously (4.2.4). DNA templates from well-characterized isolates (Table 4-1), as well as distilled water, served as PCR controls.

**5.2.6 DNA sequencing analysis.** Sequencing was performed on 371 bp PCR products on both the forward and reverse strands, on a SpectruMedix model SCE 2410 (Spectru Medix, State College, PA) automated sequencer, incorporating the ABI Big Dye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems, Foster City, CA). Electropherograms of generated sequences were inspected with Chromas software version 1.45 (Technelysium Pty. Ltd., Helensvale, Queensland, Australia). Obtained DNA sequences were analyzed with BLAST software (1). A PCR product obtained from *P. aeruginosa* GW-1 was used as a sequencing control.

### 5.3 Results.

**i) Real-time PCR and melting curve analysis.** *P. aeruginosa* clinical isolates retrieved from departmental cryo-archives from 1998 – 2001, originated from a variety of specimens including endotracheal aspirates, urine samples, pus swabs, blood cultures, tissue biopsies, and intravenous catheters. Specimens originated from teaching hospitals associated with the Department of Medical Microbiology, University of Pretoria, South Africa, serving mainly the clinical disciplines of Internal Medicine, Surgery, Pediatrics, Neurosurgery, Orthopedics and Gynecology. Although varying zone size diameters were observed, all clinical isolates ($n$ = 100) were resistant to ceftazidime in the disc-diffusion assay (data not shown). Rapid PCR revealed 83 clinical isolates to exhibit DNA template amplification in real-time with

continuous fluorescence monitoring in channel F2. Melting curve analysis revealed 82 of the 83 clinical isolates to exhibit a mean *Tm* of 55.68 ± 0.33 ºC, whilst one clinical isolate exhibited a *Tm* of 66.83 ºC (Table 5-3). Mean *Tm* values of 55.25 ± 0.30 ºC, 64.07 ± 0.72 ºC and 64.78 ± 0.59 ºC, were obtained by analysis of amplification products from $bla_{GES-2}$, $bla_{GES-1}$ and $bla_{IBC}$ control isolates respectively. Electrophoresis of amplicons obtained from well-characterized isolates (Table 4-1), all exhibited a 689 bp $bla_{GES/IBC}$ gene product except the *E. coli* ATCC 25922 isolate (data not shown). No DNA template amplification was observed in real-time with distilled water controls.

**ii) Nested-PCR detection of $bla_{GES/IBC}$.** First round PCR amplification resulted in 51 of the clinical isolates exhibiting an 864 bp amplicon. These isolates corresponded with the $bla_{GES-2}$ producing organisms described previously (section 4.3). Second round nested-PCR amplification detected an additional 37 isolates, exhibiting a 371 bp product. Thus from a 100 *P. aeruginosa* clinical isolates, 88 tested positive for a $bla_{GES/IBC}$ gene product (Table 5-3). Amplicons 864 and 371 bp in size were obtained from each control strain during first and second amplification rounds respectively, except from the negative control, *E. coli* ATCC 25922.

**iii) Sequence analysis of nested-PCR products.** Analysis of 371 bp amplification products obtained from clinical isolates resulted in 87 sequences corresponding to that of $bla_{GES-2}$ and one sequence being homologous with $bla_{GES-1}$. These results corresponded with results obtained from melting curve analysis on the LightCycler, with $bla_{GES-2}$ products exhibiting a mean *Tm* of 55.68 ± 0.33 ºC. The $bla_{GES-1}$ product corresponded with a *Tm* of 66.83 ºC. In addition to these findings, a stem loop in the secondary DNA structure (nt 466 – 470 and 482 – 486 of the coding region) was noted.

**Table 5-3: Results obtained with nested-PCR, real-time PCR and DNA sequencing methods.**

| Method | Isolate type | *bla*$_{GES/IBC}$ amplification positive | *bla*$_{GES/IBC}$ amplification negative | Number of amplicons analyzed | Tm (°C) ± SD | Sequencing result |
|---|---|---|---|---|---|---|
| **Nested-PCR** | Clinical isolates [a] | 88 | 12 | 87 | - | *bla*$_{GES-2}$ |
| | | | | 1 | - | *bla*$_{GES-1}$ |
| **Real-time PCR** | Clinical isolates [a] | 83 | 17 | | | |
| **Melting curve analyses** | Clinical isolates | | | 82 | 55.63 ± 0.33 | *bla*$_{GES-2}$ |
| | Clinical isolate | | | 1 | 66.83 | *bla*$_{GES-1}$ |
| | GES-2 control isolates [b] | | | | 55.25 ± 0.3 | |
| | GES-1 control isolate [b] | | | | 64.07 ± 0.72 | |
| | IBC control isolates [b] | | | | 64.78 ± 0.59 | |

[a] All isolates tested resistant to ceftazidime.
[b] Control isolates are shown in Table 4-1.

### 5.4 Discussion.

Multi-drug resistant *P. aeruginosa* has become a major burden in the care of long-term debilitated intensive care unit patients, affecting a wide spectrum of clinical disciplines both in the public and private sector hospitals in the Pretoria region, South Africa. Although routine detection of ESBL production in *Enterobacteriaceae* is well described (9), it is not easily accomplished with *P. aeruginosa.* In the case of GES-2 - producing *P. aeruginosa* isolates, it can be extremely difficult and time consuming in the clinical microbiology laboratory (20). The aim of this study was to develop a real-time PCR assay to reliably detect the ESBL GES-2, partially compromising the efficacy of imipenem in South African *P. aeruginosa* clinical isolates. For this, the ultra rapid LightCycler, based on real-time fluorimetric measurement of amplification products, proved ideal to analyze the "hot spot" area in the $bla_{GES}$ gene for resistance mutations as previously described with *Candida albicans* isolates (8). Previous real-time PCR work conducted on the detection of SHV-type ESBLs from *Enterobacteriaceae* clinical isolates (18), reported a 100 % sensitivity and specificity for the described LightCycler assay. During this study, the LightCycler and nested-PCR assays detected a $bla_{GES/IBC}$ product from 83 and 88 clinical isolates respectively, exhibiting a sensitivity of 94.3% for the LightCycler compared to nested-PCR. Comparison of real-time amplicon *Tm* determinations with sequencing data however revealed 100% specificity, therefore concurring with the previous study (18), with regard to the usefulness of the LightCycler for accurate identification of point mutations in ESBL genes. The noted decrease in sensitivity may be influenced by a number of factors including the quality of DNA template used in experiments (20) and the choice of both DNA polymerase and polymerase buffer systems utilized in the LightCycler assay (21).

Design and placement of the fluorogenic probes proved to be problematic due to the presence of the stem loop structure situated in close proximity upstream from the mutation site (Figure 5-1). This was overcome by placing the shorter sensor probe directly alongside the stem loop structure and designing a reverse primer that binds well away downstream from the mutation site, while still keeping the resulting amplicon short. A thorough initial denaturation step in the amplification protocol further facilitated probe and template binding prior to primer extension and amplification. Based on a previous study which demonstrated a *ca.* 6ºC temperature shift per nt mismatch (18), and due to the two nt mismatch between $bla_{GES-2}$ and the sensor probe, a *ca.* 10 – 12 ºC difference in *Tm* was expected between $bla_{GES-2}$ and $bla_{GES-1\ /\ IBC}$ amplification products respectively. However, *Tm* analyses of $bla_{GES-2}$ and $bla_{GES-1}$ products obtained from control isolates demonstrated a difference of 7.8 – 9.84 ºC, whereas $bla_{GES-2}$ and $bla_{IBC}$-type products exhibited a temperature difference of 8.64 – 10.42 ºC. Melting point analysis of 83 $bla_{GES-2}$ clinical isolates exhibited a *Tm* of 55.63 ± 0.33 ºC, clearly distinguishing the two nt mismatch from $bla_{GES-1}$ type amplification products (Figure 5-2).

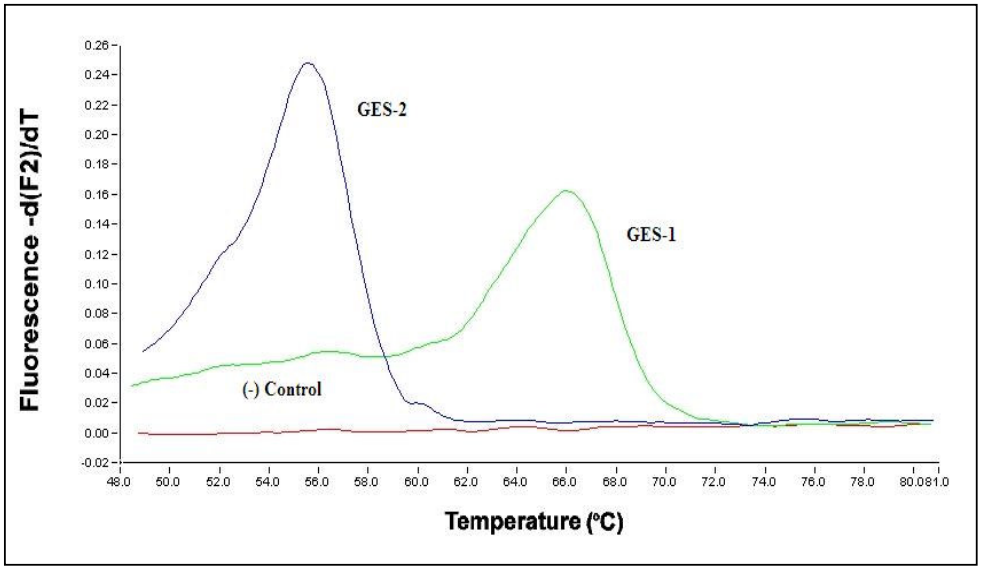


**Figure 5-2:** Melting peaks of *bla*$_{GES-1}$, *bla*$_{GES-2}$ and *E. coli* ATCC 25922 (*bla*$_{GES/IBC}$ template negative control), amplification products plotted as the negative derivative of fluorescence F2 [*-d(F2)/dt)*] versus temperature [T]. The *Tm* difference between an exact sensor probe match (GES-1) and a two-nucleotide mismatch (GES-2) is clearly visible. No melting peak was generated with the negative control isolate.

Where there was an exact match between the sensor probe and the template sequence, a *Tm* of 64.07 ± 0.72 °C (*bla*$_{GES-1}$) and 64.78 ± 0.59 °C (*bla*$_{IBC}$) was found, in comparison with 66 °C reported previously (18). These differences in *Tm* could most probably be ascribed to the genetic divergence between *bla*$_{SHV}$- and *bla*$_{GES\ /\ IBC}$-type genes (20) as well as the GC content differences evident between *bla*$_{GES-2}$ and the *bla*$_{GES-1\ /\ IBC}$ mutation areas examined by this study (17). Future research incorporating real-time PCR detection of the *bla*$_{GES}$ genes should investigate the efficacy of silica based DNA extraction to improve template purity (3), as well as the development of fluorogenic probes that can cost-effectively categorize this growing family of ESBL genes into clinically relevant subgroups (18).

One clinical isolate with a *Tm* of 66.83 °C, proved to be a $bla_{GES-1}$ product on sequence analysis, making this the first report of beta-lactamase GES-1 from a South African clinical isolate of *P. aeruginosa*. This isolate was collected in 1999 originating from a blood culture from a 38 year old, male patient in a surgical ICU after being treated for multi-trauma complicated by a nosocomial pneumonia. On treatment with parenteral imipenem he made a subsequent full recovery. The collection of this GES-1-producing isolate almost coincides with the original description of $bla_{GES-1}$ from a *K. pneumoniae* clinical isolate originating from French Guiana in South America (16). This data finally proves that both GES-1 and GES-2 ESBL-producing *P. aeruginosa* circulate in Pretoria, South Africa, with GES-2 being possibly more advantageous to, and prevalent in, this species.

Until such time as the NCCLS and other laboratory standards organizations formulate criteria for the detection of ESBLs from *P. aeruginosa* and other non-fermentative bacterial species, this phenomenon will go by mostly undetected in the routine laboratory with possible dire clinical consequences. Currently, molecular detection of ESBL encoding genes from these species are the only reliable method available and as such, the LightCycler has proved to be sensitive and highly specific in the detection of ESBL genes of the GES / IBC family from *P. aeruginosa.* Further developments in ESBL production from non-fermenting Gram-negative bacteria will certainly necessitate the use of a fast and dependable molecular diagnostic method, to possibly enhance the diagnostic capabilities of the clinical microbiology laboratory in this regard.

**5.5 References.**


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