CHAPTER 4: SEQUENCE-SELECTIVE RECOGNITION OF EXTENDED-SPECTRUM BETA-LACTAMASE GES-2, BY A COMPETITIVE, PEPTIDE NUCLEIC ACID BASED, MULTIPLEX-PCR ASSAY.

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4.1 Introduction

After the discovery of the extended-spectrum beta-lactamase (ESBL), GES-2 from *Pseudomonas aeruginosa*, in Pretoria, South Africa in the year 2000 (24), it became clear that class 1 integron borne ESBLs were established in the South African nosocomial setting. The same strain subsequently caused a nosocomial outbreak with diverse clinical presentations in three Intensive Care Units (ICUs) in a teaching hospital, during which several patients succumbed (25). The *P. aeruginosa* strain described during that outbreak exhibited a tendency to colonise and infect mostly debilitated patients, including human immunodeficiency virus (HIV)-positive patients, significantly increasing both their length of stay in the ICU and cost of treatment (25). Detection of GES-2 and other ESBL-producing *P. aeruginosa* isolates in the clinical microbiology laboratory utilising ordinary methods is notoriously difficult due to various reasons including: i) false negative results due to naturally occurring beta-lactamases such as the chromosome-encoded AmpCs that may be over-expressed, ii) simultaneous presence of metallo-enzymes with carbapenem-hydrolysing activity (IMP and VIM series), or with extended-spectrum oxacillinases (OXA-2 and OXA-10 derivatives), iii) relative resistance to inhibition by clavulanate as exemplified by GES-2, and iv) combined mechanisms of resistance such as impermeability and efflux (29). When an ESBL is suspected in *P. aeruginosa*, polymerase chain reaction (PCR)-based molecular techniques may help to detect the gene with a series of primers designed for recognition of class A beta-lactamase

genes in this species (29). However, PCR experiments without further sequencing of the amplification products could not accurately differentiate between all the genes in a beta-lactamase family, substantially adding to the costs of such an exercise. Other methods such as isoelectric focusing analysis (IEF) may just indicate the presence of acquired beta-lactamases rather than to identify an ESBL precisely (18, 29). Descriptions of $bla_{\mathrm{GES-1}}$ (5, 26) and the bla_{IBC}-type genes (8, 13) from different geographical locations further complicates the laboratory identification of $bla_{\mathrm{GES-2}}$, the more potent and currently geographically restricted gene in this family (24, 29). Since the initial description of peptide nucleic acid (PNA) in 1991 (17), interest in this synthetic polymer as a molecular device has steadily increased. The hallmark of PNA that stimulated this interest is its intrinsic high affinity and specificity for complementary nucleic acids (17), making it the ideal nucleic acid molecule to recognise point mutations without the need for highly specialised equipment. The fact that the GES / IBC beta-lactamase family is still relatively small and with only a few point mutations separating the genes in question (29), makes it the ideal model for the development of a novel sequence-specific, PNA-based identification method for geographically restricted ESBLs, suited to large scale, low-cost screening programmes.

4.2 Materials and methods

4.2.1 Bacterial strains. One hundred clinical isolates of *P. aeruginosa*, collected and cryo-preserved by the Department of Medical Microbiology, University of Pretoria, South Africa, were included in this study. Isolates were identified with the API-20NE system (bioMérieux, Marcy l’Etoile, France) according to the manufacturer’s instructions. Control bacterial strains used in this study to standardize reactions are listed in Table 4-1.

Table 4-1: Well-characterized bacterial strains used in this study.

Strain	Relevant properties	Source or reference
P. aeruginosa GW-1	*bla*$_{GES-2}$ producing isolate	24
P. aeruginosa Pu21	*bla*$_{GES-2}$ transconjugant isolate	24
K. pneumoniae ORI-1	*bla*$_{GES-1}$-producing isolate	26
E.cloacae HT-9	*bla*$_{IBC-1}$-producing isolate	8
E. coli brcpHT-8	*bla*$_{IBC-1}$ transconjugant isolate	8
E.coli DH5α	*bla*$_{IBC-2}$ transconjugant isolate	13
E. coli ATCC 25922	*bla*$_{GES}$ / *bla*$_{IBC}$ negative strain	ATCC[a]

[a] ATCC, American Type Culture Collection, Manassas, VA.

4.2.2 Susceptibility testing. Antibiotic-containing disks (Mast Diagnostics, Merseyside, UK) were used for routine laboratory antibiograms by the disk diffusion assay as described and interpreted, according to National Committee for Clinical Laboratory Standards (NCCLS) guidelines (15). Resistance to ceftazidime (MIC ≥ 32 µg/ml) (15), was an absolute requirement for inclusion in the study.

4.2.3 DNA extraction. Extraction of whole-cell DNA was performed with a precipitation-based method as described previously (23, Appendix A) combined with an added double ethanol final wash step to improve template purity (9). DNA pellets were dried in a DNA Speed Vac 110 (Savant Instruments Inc., Farmingdale, NY) and resuspended in 1 ml TE buffer (10 mM Tris/HCl, 1mM EDTA, pH 7.4). DNA extraction from each isolate was verified by means of electrophoresis at 2 V/cm for 1 hour in 0.8% agarose gel containing ethidium bromide 0.5 µg / ml in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 7.4) and visualised under UV light.

4.2.4 Standard PCR amplification.

i) Standard PCR primer construction. A primer pair annealing internally to the binding sites of previously published primers GES-1A and GES-1B (24), was constructed from the gene sequence of bla_{GES-2} [GenBank accession number AF 326 355, (24)], by utilising Primer3 software (Whitehead Institute for Biomedical Research) available from URL: http://www.inqaba.com. The following primer sequences were generated: GES-C, 5`-GTT TTG CAA TGT GCT CAA CG-3` (positions 176 – 195 of the coding region) (*Tm* = 54.6ºC) and GES-D, 5`-TGC CAT AGC AAT AGG CGT AG-3` (positions 527 – 546) (*Tm* = 55.6ºC)(Table 4-2), targeting a 371 base pair (bp) region, incorporating all currently known point mutations of bla_{GES} and bla_{IBC} genes (5, 8, 13, 24, 26). Primers were synthesized and purified by Integrated DNA Technologies Inc., Coralville, Iowa.

ii) Standard PCR amplification and detection. GES-C and GES-D primers were utilized under standard PCR conditions (28), to amplify a 371 bp product. The 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 deoxynucleotide triphosphate, 1.25 U *Taq* DNA polymerase, (Promega Corp., Madison, WI), 2 µL DNA template and distilled water to a final reaction volume of 50 µL. Amplification was conducted on a GeneAmp PCR system 9600 (Perkin Elmer Cetus, Emeryville, CA) and consisted of an initial denaturation step at 95°C for 2 min, followed by 35 amplification cycles each comprising a denaturation step at 95°C for 30 sec, followed by an annealing step at 50°C for 1 min, and an extension step at 72°C for 1 min. Completion of 35 amplification cycles was finally followed by an extension step at 72°C for 5 min. PCR products were visualized by electrophoresis at 4 V/cm for 45 min in 0.8% agarose gel containing ethidium bromide 0.5 µg/ml. Amplicon size was verified with a 100 bp

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DNA ladder (Promega Corp.). DNA templates obtained from *P. aeruginosa* GW-1, *bla*$_{GES-2}$ transconjugant *P. aeruginosa* Pu21, *K. pneumoniae* ORI-1, *Enterobacter cloacae* HT-9, *bla*$_{IBC-1}$ transconjugant *E. coli* brcpHT-8 and *bla*$_{IBC-2}$ transconjugant *E. coli* DH5α (Table 4-1), served as PCR positive controls. *E. coli* ATCC 25922 was used as a *bla*$_{GES/IBC}$ PCR negative control. All PCR experiments were performed in duplicate. Reaction preparation was done in separate rooms equipped with laminar flow cabinets to avoid cross-contamination with amplification products.

Table 4-2: Oligonucleotide sequences used in this study.

Primer / probe	Sequence [a]	Function	Source or reference
Standard PCR and DNA sequencing			
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
PNA-based multiplex PCR			
GES-1A	ATG CGC TTC ATT CAC GCA C	Forward primer	24, 29
GES-1B	CTA TTT GTC CGT GCT CAG G	Reverse primer	24, 29
GES-E	GTG TGT TGT CGT TCA TCT C	Reverse primer – complementary to *bla*$_{GES-2}$	This study
GES-F	CCT GGC GAC CTC AGA GAT AC	Forward primer	This study
PNA probe	GTT GTC GCC CAT CTC	Complementary to *bla*$_{GES-1}$ / *bla*$_{IBC}$	This study

[a] – Sequences are delineated in 5` - 3` order.
Relevant point mutations are depicted in shaded boxes.

4.2.5 Competitive PNA-based multiplex PCR. This novel method to detect point mutations in ESBL genes by PCR relies on two features of PNA: i) its intrinsically

high affinity for DNA (17) and ii) its inability to serve as a primer for DNA polymerases (21). This method utilizes a forward primer in conjunction with a reverse primer that is complementary to the desired mutant sequence, as well as a PNA probe similar to the reverse primer, but complementary to the wild type (wt) sequence. When the reaction contains a wt DNA template, the PNA probe out competes the reverse primer for binding and no amplification occurs, conversely the opposite happens when a mutant sequence is present during the reaction (20). A second set of primers targets an area downstream from the mutation site in both wt and mutant genes; this then serves as an internal amplification control measure.

i) Multiplex PCR primer construction. In addition to primers GES-1A and GES-1B (24), primers for multiplex PCR analysis were constructed in a similar fashion as described for the standard PCR method. The following primer sequences were generated: GES-E, 5`-GTGT GTT GTC GTT CAT CTC-3` (positions 487 – 505 of the $bla_{\text{GES-2}}$ coding region) (Tm = 51.8ºC) and GES-F, 5`- CCT GGC GAC CTC AGA GAT AC-3` (positions 505 – 524) (Tm = 57.4ºC) (Table 4-2).

ii) Peptide nucleic acid probe construction. A mixed sequence PNA probe was designed to be complementary to positions 487 – 501 of the coding regions of $bla_{\text{GES-1}}$, bla_{IBC1} and bla_{IBC2} [GenBank accession numbers AF 156486, AF 208529 and AF 329699, (8, 13, 26)] utilizing Custom PNA Probe Designer software (Applied Biosystems, Rotkreutz, Switzerland) and available from URL: http://www.appliedbiosystems.com. The 3` L-lysine-labeled probe sequence in antiparallel binding mode (20), 5`-GTT GTC GCC CAT CTC-3` (Tm = 70ºC), was synthesized and purified by Boston Probes, Bedford, MA.

iii) Reaction preparation and conditions. In a multiplex-PCR assay, primers GES-1A with GES-E and GES-F with GES-1B were used to amplify 505 and 360 bp

products respectively. The 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 deoxynucleotide, 1.25 U *Taq* DNA polymerase, (Promega Corp.), 2 μL DNA template and distilled water to a final reaction volume of 50 μL. Amplification and detection was performed as described for the standard PCR procedure previously. Once the reaction conditions were standardized with well-described bacterial isolates (Table 4-1), PNA probe to a final concentration of 0.32 μM were added to subsequent multiplex PCR reactions to compete with the reverse primer GES-E.

4.2.6 DNA sequencing analysis. Sequencing was performed on standard PCR products on both the forward and reverse strands, on a SpectruMedix model SCE 2410 automated sequencer (Spectru Medix, State College, PA), 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 the BLAST program (1), available from URL: http://www.ncbi.nlm.nih.gov. A PCR product obtained from *P. aeruginosa* GW-1 (Table 4-1), was used as a control.

4.3 Results

i) Detection of *bla*$_{\mathbf{GES/IBC}}$ with standard PCR. Clinical isolates of *P. aeruginosa* collected and cryo-preserved from 1998 – 2001 by the Department of Medical Microbiology, University of Pretoria, South Africa, originated from a variety of specimens including endotracheal aspirates, urine samples, pus swabs, blood cultures, tissue biopsies, and intravenous catheters. Specimens originated mainly from ICUs, serving Internal Medicine, Surgery, Pediatrics and Neurosurgery, while the remainder

of specimens originated from general hospital wards including orthopedic and gynaecology patients. (For the purposes of this study, clonal relatedness of isolates was not determined.) Although varying zone size diameters were observed, all isolates (*n* = 100) were resistant to ceftazidime (MIC ≥ 32 μg/ml) in the disc-diffusion assay according to NCCLS guidelines (15) (data not shown). Repeated standard PCR amplification resulted in 51 of the isolates testing positive for a *bla*$_{GES / IBC}$ gene product comprising 371 bp in length. A 371 bp PCR product was obtained from each control strain (Table 4-1), except from the *bla* $_{GES / IBC}$ negative control, *E. coli* ATCC 25922.

ii) DNA sequencing of standard PCR products. By sequence analysis of the standard PCR products (*n* = 51) on both the forward and reverse strands, all products corresponded to *bla*$_{GES-2}$. Each sequence was thoroughly inspected for signature point mutations, such as the 5`-GCT-3` motif (positions 358 – 360 of the coding region), differentiating *bla*$_{GES}$– from *bla*$_{IBC}$-type genes (26) and the 5`-GAA-3` motif (positions 492 – 494), differentiating *bla*$_{GES-2}$ from *bla*$_{GES-1}$ and *bla*$_{IBC}$-type genes (24). In addition to these specific motifs as mentioned above, a stemloop in the secondary DNA structure (positions 466 – 470 and 482 – 486 of the coding region) was noted.

iii) PNA-based, sequence specific detection of *bla*$_{GES-2}$. Subjecting the 100 clinical isolates to sequence-specific multiplex-PCR amplification resulted in 50 of the 51 standard PCR positive isolates, testing positive for *bla*$_{GES-2}$. A repeat multiplex-PCR reaction with DNA template volume increased from 2 – 5 µl, yielded a positive result for the outstanding isolate. Detection of amplification products by gel electrophoresis exhibited two distinctive patterns, GES-2-producing clinical isolates and control strains producing two distinct bands of 505 and 360 bp respectively, while GES-1-

and IBC-producing control strains only produced the 360 bp internal amplification control band, clearly distinguishing them from GES-2 producing isolates (Figure 4-1).

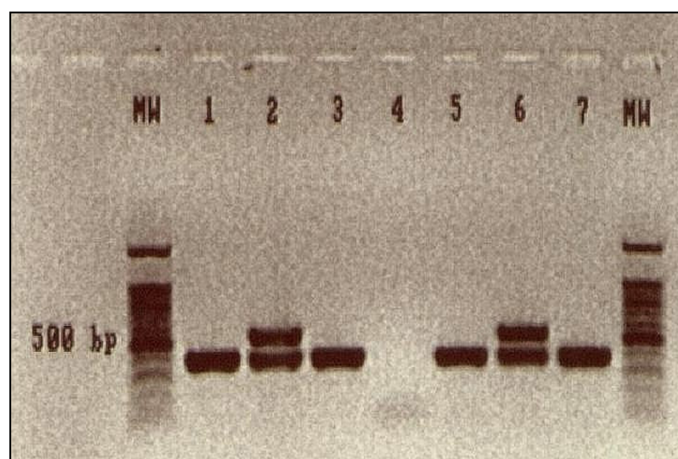

Figure 4-1: Gel electrophoresis depicting PNA-based, sequence-specific PCR amplification products obtained from well-characterized bacterial isolates listed in Table 4-1. MW: 100 bp marker (Promega Corp.), 500 bp segment indicated; lane 1: *E. cloacae* HT-9; lane 2: *P. aeruginosa* GW-1; lane 3: *E. coli* brcpHT-8; lane 4: *E. coli* ATCC 25922; lane 5: *K. pneumoniae* ORI-1, lane 6: *P. aeruginosa* Pu21; lane 7: IBC-2 transconjugant *E. coli* DH5α.

Control strains *P. aeruginosa* GW-1 and *P. aeruginosa* Pu21 (24) both tested positive for *bla*$_{GES-2}$ in repeated reactions, while repetitive results for *bla*$_{GES-1}$ and *bla*$_{IBC}$-producing strains (Table 4-1) were obtained. The *E. coli* ATCC 25922 isolate tested negative in all reactions. These results corresponded with the results obtained for the standard PCR amplification and sequencing reactions.

4.4 Discussion

Despite considerable effort from various investigators, detection of ESBLs in *P. aeruginosa* still remains a problem due to the notoriously low sensitivities of easy-to-perform susceptibility tests or to the laborious and often expensive molecular

detection methods (29). 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 (12), PCR-restriction fragment length polymorphism analysis (2, 19), single-strand conformation polymorphism analysis (14), real-time PCR with melting curve analysis (27), and ligase chain reaction-based tests (10, 16). In addition to these aforementioned methods, this study describes the application of PNA as a novel method to detect point mutations in ESBL genes of the GES / IBC family.

Data from studies with nuclear magnetic resonance (NMR) (11) and X-ray crystallography (3) have shown that mixed sequence PNA forms duplexes with complementary DNA sequences by way of base specific hydrogen bonds, with a 1:1 stoichiometry, obeying Watson-Crick hydrogen bonding rules (7). Hybridization properties of mixed sequence PNA and consequently the high affinity of the interaction of PNA with DNA can be attributed to the lack of negative charge in the PNA backbone as demonstrated by melting point analysis at high ionic concentrations (> 1 M Na^+) (7). Previous analysis of PNA base mismatch recognition in DNA templates have shown that from the 12 possible single base mismatches, all but one (G_{PNA} and T_{DNA}) had a larger destabilizing effect on the PNA-DNA duplexes than on the corresponding DNA-DNA duplexes, regardless of the ionic concentration of the medium (22). The two bp mismatch targeted in this study (CC_{PNA} and AA_{DNA}), provided sufficient instability of the PNA-DNA duplex in the presence of bla_{GES-2} (mutant) templates to facilitate replacement of the PNA probe by the reverse primer GES-E, in order for amplification to occur (Figure 4-2). In the presence of bla_{GES-1} (wild type) or bla_{IBC}-type templates, the resulting PNA-DNA duplex experienced no mismatch and was therefore stable enough to prevent binding of the reverse primer,

henceforth disabling PCR amplification in a sequence specific manner. These data coupled with the ability of PNA to be sequence specific regardless of ionic concentration during the PCR reaction (7, 22), makes it a very robust molecular tool, well suited for screening large numbers of isolates for very specific point mutations. Sensitivity and specificity of this novel method to accurately detect the GG to AA point mutation in $bla_{\mathrm{GES\text{-}2}}$, matched those generated by the "gold standard" (PCR and DNA sequencing) perfectly. As published previously, high quality of the DNA template is however imperative to avoid false negative results (29), as was noted with one of the clinical isolates when subjected to the sequence-specific multiplex-PCR method. Large-scale epidemiological use of this novel PNA-PCR method would possibly require the use of a commercially available DNA extraction process, to standardize DNA template yield and input. The addition of a second primer pair, targeting a 360 bp region downstream from the mutation site has therefore been incorporated into the reaction to serve as an internal amplification control. The presence of a stemloop in the secondary structure of the bla_{GES} and bla_{IBC} DNA templates used in this study was noted (CGGCT and AGCCG motifs situated at positions 466 – 470 and 482 – 486 of the coding region)(Tm = 82ºC), creating a possible steric hindrance during annealing of the reverse primer GES-E to the closely situated target site. This was overcome by a thorough initial denaturation step in the amplification protocol and permitting the PNA probe to bind earlier to the target sequence relative to the competing reverse primer, due to the rapid formation of antiparallel PNA-DNA duplexes in comparison with duplexes formed in parallel binding mode (20).

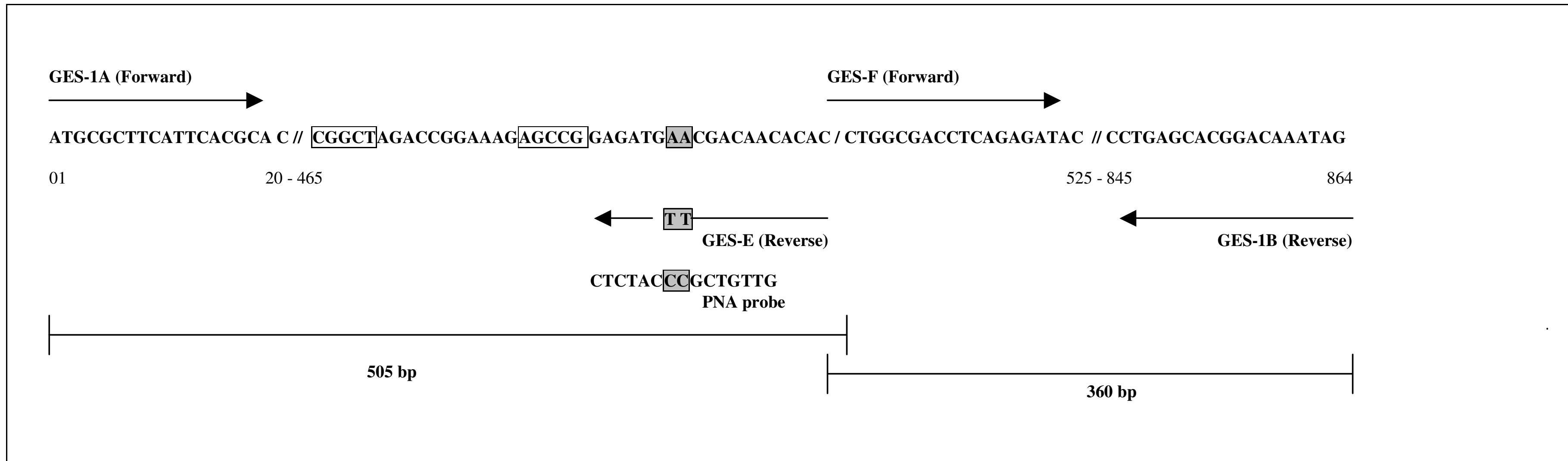

Figure 4-2: Schematic diagram depicting the placement of amplification primers and the competitive PNA probe relative to the gene sequence of *bla*$_{GES-2}$, (nt 1 – 864). Relevant point mutations are exhibited in shaded boxes, whereas stemloop sequences are depicted in open boxes. Note the CC_{PNA} - AA_{DNA} nucleotide mismatch, causing instability of the PNA-DNA duplex, thereby facilitating replacement of the PNA probe with the reverse amplification primer GES-E. Amplification products (505 and 360 bp respectively) are depicted by straight lines. Sequence direction is delineated as 5` - 3`.

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Additionally, by constructing the PNA probe to bind in a highly thermostable, antiparallel mode to the complementary DNA target (20), the resulting PNA-DNA duplex formation impaired the amplicon interstrand reassociation (6), thereby further stabilizing the reverse primer-binding site on the DNA template.

Novel methods to detect and identify genes encoding ESBLs will continue to develop as our understanding of molecular methods increase. However, the most powerful driving mechanism behind this movement will most probably be to stay within budgetary restraints. The remarkable ability of PNA to hybridize in a sequence-specific manner with high affinity to complementary nucleic acids and thereby acting as a PCR controller, makes it the ideal molecule to use in screening programmes for well-characterized, specific or geographically restricted ESBLs. This method will however not detect point mutations outside of the chosen mutation site and therefore only offers a partial alternative to gene sequencing. Proper modification of this method to suit individual needs may prove to be highly cost effective in resource poor settings, not only from savings incurred by direct detection of epidemiologically relevant point mutations, but also through not having to procure expensive equipment.

4.5 References

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