CHAPTER 6: GENETIC STABILITY OF CLASS 1 INTEGRON-BORNE, *BLA*$_{GES}$-TYPE GENES UNDER SHORT-TERM, SELECTIVE, IN-VITRO ANTIBIOTIC PRESSURE.

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6.1 Introduction

The emergence of the novel GES family of extended-spectrum beta-lactamases (ESBLs) belonging to Ambler class A, has been demonstrated in South America, Europe, South Africa and Japan recently (1, 6, 15, 16, 20, 21, 22). In addition to hydrolyzing various beta-lactam antibiotics, including several expanded-spectrum cephalosporins, weak carbapenem hydrolysis and relative resistance to beta-lactamase inhibitors have been demonstrated previously in GES-2 (15). This expansion of substrate profile, compared to beta-lactamase GES-1, was ascribed to the G170N substitution in the omega loop of the mature protein (15, 16). Recent reports from Japan (21) and Greece (20) respectively, describe amino acid substitutions in Ambler position 170 of the omega loop region of novel enzymes belonging to the GES family. A single G170S substitution as reported for GES-4 is most likely associated with more efficient hydrolysis of carbapenems and cephamycins as well as exhibiting a decreased affinity for beta-lactamase inhibitors (21). Structural changes to the omega loop, as hypothesized previously, may be involved in increased binding of the hydroxy-ethyl moieties of carbapenems (12). In recent reports describing novel point mutations in GES-type genes, selective antibiotic pressure is thought to be a major factor, facilitating mutation and possible dissemination in the nosocomial environment (20, 21). In this study, the short-term, continuous exposure, of two well-described *bla*$_{GES}$–producing isolates to sub-inhibitory levels of single antimicrobial

agents, was examined to determine the possible effects on these genes and their immediate upstream regulatory environments.

6.2 Materials and methods

6.2.1 Bacterial strains. Bacterial isolates *Klebsiella pneumoniae* ORI-1 (16) and *Pseudomonas aeruginosa* GW-1 (15) were used in this study. Both were initially described as clinical isolates and natural carriers of class 1 integron-borne bla_{GES-1} and bla_{GES-2} respectively. In addition, both were found to be resistant to various antibiotics as reported previously (15, 16), and were suitable to imitate local clinical conditions closely. Isolates were cryo-preserved at -75˚C prior to this study (17).

6.2.2 Antibiotic challenge assay. Fifteen different antibiotic containing solutions were made up in sterile Mueller-Hinton broth (Becton Dickinson Co, Cockeysville, MD) with concentrations corresponding to sensitive breakpoints for each species as specified by the National Committee for Clinical Laboratory Standards (NCCLS) or as previously published (7, 14) (Table 6-1). Antimicrobial agents were obtained from Abtek Biologicals Ltd., Liverpool, UK, Merck Sharpe & Dohme (Pty) Ltd, Halfway House, South Africa (imipenem) and AstraZeneca Pharmaceuticals (Pty) Ltd, Sunninghill, South Africa (meropenem) and reconstituted according to the recommendations of the respective manufacturers. Commonly used antibiotics in local training hospitals were chosen for evaluation during this study, based on data obtained during a recent departmental audit (Department of Medical Microbiology, University of Pretoria – 2004). Inoculae of *K. pneumoniae* ORI-1 and *P. aeruginosa* GW-1 were prepared by the growth method in broth medium as described elsewhere (14). On day one, 100 µl of this cell suspension was then inoculated into 10 ml of each antibiotic solution (Table 6-1) and incubated under constant shaking at 37˚C for

24 hours. On day two, 100 µl of the day one culture was passaged to a corresponding freshly prepared 10 ml antibiotic solution and incubated as described.

Table 6-1: Antibiotics and antibiotic concentrations used in this study.

Antibiotic	***K. pneumoniae* ORI-1 (µg/ml)**	***P. aeruginosa* GW-1 (µg/ml)**
Ampicillin	8	8[a]
Piperacillin-Tazobactam	16/4	64/4
Cephazolin	8	8[a]
Cefuroxime	8	8[a]
Ceftazidime	8	8
Meropenem	4[c]	4
Imipenem	4[c]	4
Nalidixic acid	16	16[a]
Ofloxacin	2	2
Ciprofloxacin	1	1
Amikacin	16	16
Gentamicin	4	4
Trimethoprim - sulfamethoxazole	2/38	2/38
Colistin sulphate	1[b]	1[b, c]
Nitrofurantoin	32	32[a]

[a] – Antimicrobial breakpoints for *Enterobacteriaceae* were used for *P. aeruginosa* isolates where none existed (14).
[b] – Colistin sulphate concentrations were reconstituted as previously published (15).
[c] – Isolates did not survive beyond 24 hours of incubation and were excluded from further analyses.

This process was repeated for each isolate until five days of incubation (120 hours) was reached. The same process was followed with controls, which were inoculated

into sterile, antibiotic-free Mueller-Hinton broth (Becton Dickinson Co). Cells were then harvested from each solution by centrifugation (4500 x g, 10 min, 4˚C) and the cell pellet was washed twice in 1 ml distilled water by vortexing (2500 rpm, 10 s) and centrifugation (4500 x g, 10 min, 4˚C). Washed cell pellets were resuspended in sterile liquid broth [1ml Brain-heart infusion broth (Oxoid Ltd., Hampshire, UK) with 10% v/v glycerol added (Merck, Darmstadt, Germany)] and stored at -75˚C (17), until further analysis. Cryopreserved cells were revived on a non-selective, antibiotic free, nutrient agar medium (Oxoid Ltd.) for 24 hours, incubated at 37˚C in ambient atmosphere, prior to further analysis.

6.2.3 DNA extraction. Extraction of whole-cell DNA was performed with an ethanol precipitation-based method as described previously (23, Appendix A). DNA pellets were dried in a DNA Speed Vac 110 (Savant Instruments Inc., Farmingdale, NY), resuspended in 1ml TE buffer (10 mM Tris/HCl, 1mM EDTA, pH 7.4) and stored at 4˚C until further analysis.

6.2.4 PCR amplification and detection. A primer pair annealing to both the *IntI* and bla_{GES} genes of class 1 integrons from *K. pneumoniae* ORI-1 and *P aeruginosa* GW-1 respectively (15, 16), was designed with Primer3 software (Whitehead Institute for Biomedical Research) as described previously (23). Primers Int-E and Int-F targeted a 814 bp region from both isolates including the –10 region of promoter P_1 (3, 15), the –35 and –10 regions of P_2 (15), the *attI1* site and the core site motif GTTAGAC, situated upstream of the first gene cassette, bla_{GES} (15, 16). Primer Int-E and previously published reverse primer GES-1B (15) targeted the aforementioned structures as well as the complete sequence of the first gene cassette bla_{GES}. Primer

sequences appear in Table 6-2. Primers were synthesized and purified by Integrated DNA Technologies Inc., Coralville, Iowa. Whole-cell DNA obtained from isolates subjected to the antibiotic challenge assay served as experimental templates in a standard PCR amplification assay. PCR cycling conditions and detection of amplicons by electrophoresis are described elsewhere (23). DNA from non-exposed isolates served as controls, distilled water served as a DNA negative PCR control.

Table 6-2: Oligonucleotide sequences used in this study.

Primer name	Sequence 5` – 3`	Function	Origin or reference
Int-E	AGG ATG CGA ACC ACT TCA TC	Forward	This study
Int-F	CGG TGC CTG AGT CAA TTC TT	Reverse	This study
GES-1B	CTA TTT GTC CGT GCT CAG G	Reverse	8

6.2.5 Restriction enzyme analysis: PCR products produced with primers Int-E and Int-F (Table 6-2) were analyzed for cassette insertion into the *attI1* site (5, 10) with restriction enzyme *Ava*I (Promega Corporation, Madison, WI). *Ava*I was chosen based upon virtual restriction digests of expected template sequences performed with Restriction Enzyme Site Mapper version 3 software available from URL: http://www.restrictionmapper.org. Restriction enzyme reactions were carried out according to the directions of the manufacturer. Detection of restriction fragments was performed with field inversion gel electrophoresis (FIGE) (4V/cm, reorientation angle 180˚, pulse frequency $1s^{-1}$) in a 1% small fragment agarose gel (Promega Corp.) containing ethidium bromide (0.5 µg/ml) with TBE running buffer (45 mM Tris-

borate, 1 mM EDTA, pH 7.4) and visualized under UV light. A 100 bp DNA marker (Promega Corp.) was used for sizing of fragments.

6.2.6 DNA sequencing. Automated sequencing of PCR products obtained with primers Int-E and GES-1B (Table 6-2) was performed and analyzed as described previously (23).

6.3 Results

Standard PCR amplification. Amplicons obtained with primers Int-E and Int-F (Table 2) from *P. aeruginosa* GW-1 and *K. pneumoniae* ORI-1 isolates subjected to 5 days incubation in the presence of sub-inhibitory concentrations of antibiotics, were all ca. 800 bp in size. This correlated with PCR products obtained from non-exposed controls (Figure 6-1). Amplicons obtained with primer pair Int-E and GES-1B (Table 6-2) were all ca. 1500 bp in size, again correlating with results obtained with non-exposed controls (data not shown). No amplification product was obtained with the DNA negative control. *K. pneumoniae* ORI-1 and *P. aeruginosa* GW-1 isolates could not sustain growth beyond 24 hours of incubation in the presence of carbapenems and colistin sulphate respectively (Table 6-1), and were excluded from further analysis.

***Ava*I restriction enzyme analysis.** PCR products produced with primers Int-E and Int-F (Table 6-2) were cut with *Ava*I, 100 bp upstream of the core site motif GTTAGAC, yielding two restriction fragments of 394 and 416 bp respectively (Figures 6-1, 6-2). Cassette insertion at the *attI1* site would have significantly increased the size of the 394 bp fragment making it relatively simple to detect by electrophoresis, none of which could be demonstrated when compared to non-exposed controls.

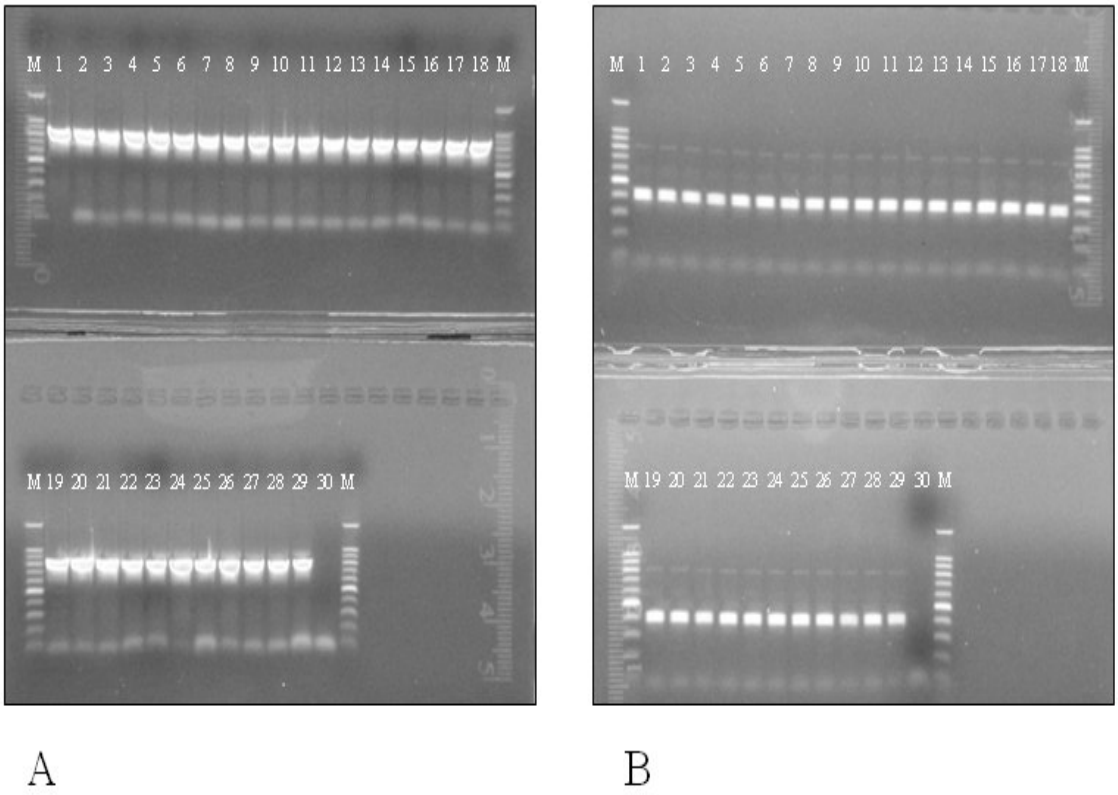

Figure 6-1: Gel electrophoresis depicting Int-EF PCR products (Panel A) and *Ava*I restriction of Int-EF PCR products (Panel B). M – 100 bp molecular marker (Promega Corp.). Lanes 1-13 *K. pneumoniae* ORI-1 exposed isolates: [1-SXT, 2-AMP, 3-COL, 4-CXM, 5-AMK, 6-NIT, 7-OFX, 8-CAZ, 9-CIP, 10-GEN, 11-NAL, 12-CFZ, 13-TZP], 14-*K. pneumoniae* ORI-1 non-exposed control. Lanes 15 – 28 *P. aeruginosa* exposed isolates: [15-NIT, 16-CFZ, 17-CAZ, 18-GEN, 19-AMP, 20-TZP, 21-CXM, 22-NAL, 23-CIP, 24-OFX, 25-AMK, 26-SXT, 27-MEM, 28-IPM], 29-*P. aeruginosa* GW-1 non-exposed control, 30-DNA negative control.

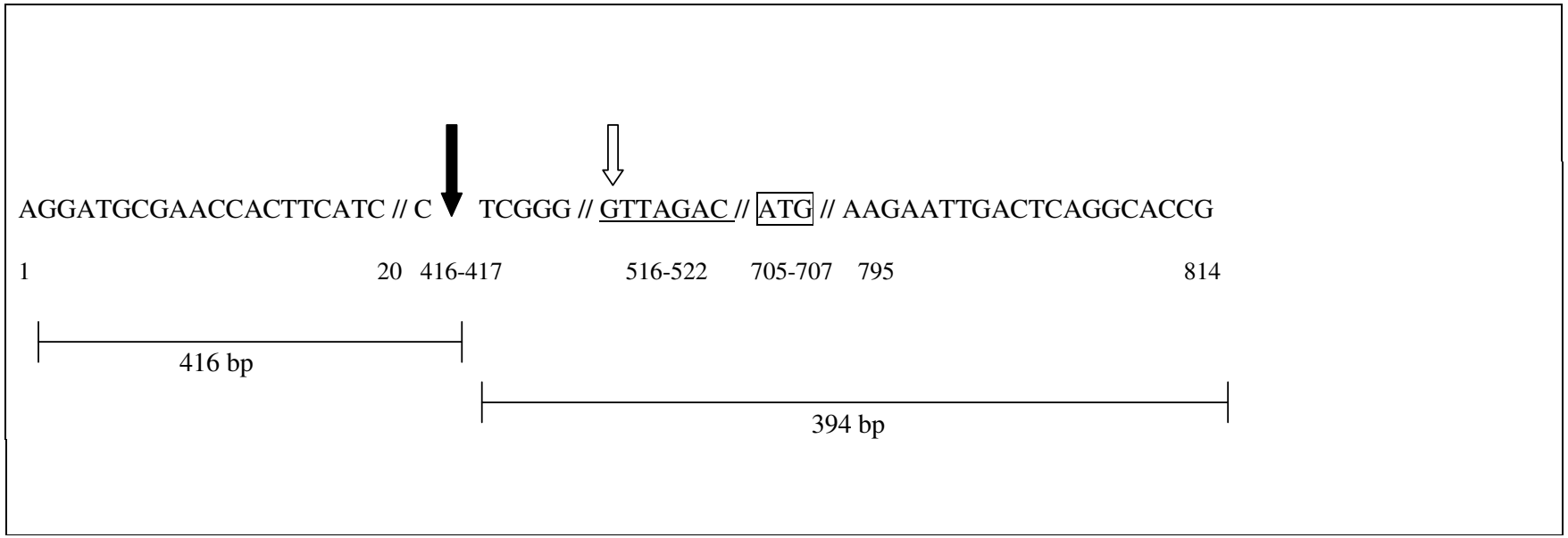

Figure 6-2: Schematic representation of *Ava*I restriction of PCR products obtained with primers Int-E and Int-F. The restriction site is marked with a black arrow (nt 416-417), the core site is underlined (nt 516-522), a white arrow indicates the recombinant crossover point (nt 516-517) and the bla_{GES} start codon (nt 705-707) is boxed. Horizontal lines depict the restriction fragment sizes of 416 and 394 bp respectively (not drawn to scale). Primers Int-E (forward) and Int-F (reverse) are depicted by nt 1-20 and 795-814 respectively.

DNA sequencing. Automated sequencing of amplicons obtained with primers Int-E and GES1-B (Table 6-2), demonstrated no difference between antibiotic exposed isolates and non-exposed controls. No differences could be detected in the promoter sequences of P_1 and P_2 compared to the previously published sequences (15, 16). The core site motif GTTAGAC upstream from *bla*$_{GES}$ remained stable, as did the nucleotide sequences of the omega loop regions of both *bla*$_{GES-1}$ and *bla*$_{GES-2}$ respectively.

6.4 Discussion

Rapid developments in the GES-type family of extended-spectrum beta-lactamases were reported lately, focusing mainly on novel point mutations leading to expansion of substrate profiles to include carbapenems (20, 21, 22). Reports originated from geographically diverse locations, suggesting that local antibiotic usage and practices may play an active role in promoting the selection of point mutations in *bla*$_{GES}$-type genes (20, 21). All GES-type genes described to date, mainly exist as gene cassettes on class 1 integrons [a notable exception was reported from Portugal (6)], with initial descriptions of *bla*$_{GES}$- type genes being situated closest to the 5` conserved segment (5`CS), containing the dual promoters P_1 and P_2 (15, 16). Very similar descriptions of the genetic environments of *bla*$_{GES-3}$ and *bla*$_{GES-4}$ originated from Japan, with GES-type genes situated closest to the promoter region (21, 22). A recent report from Greece however, details the gene cassette arrangement of *bla*$_{GES-3}$ and *bla*$_{GES-4}$ being preceded by an *aacA4* gene cassette (20), imitating integron In111 in which *bla*$_{IBC-1}$ is preceded by *aac(6`)Ib* (8, 19). Consequently, the expression level of an integron-borne antibiotic resistance gene is influenced by its position in the variable region and thus its relative distance from the common promoter region (2, 3). Major changes in

class 1 integrons, such as novel cassette integration events in the variable region, preferentially take place at the *attI1* recombination site, located at the 5`-end of the residing cassettes (3, 5, 9, 10, 11). Since *attI* sites lack the inverse core site motif RYYAAC, it cannot form palindromic structures like *attC* sites (10), therefore making it the preferred site to examine for cassette integration events. However, as demonstrated previously, where several cassettes are already present in the integron, recombinational events may occur between two 59-be sites, depending on the efficiencies of the various possible recombination reactions (4). In addition, gene cassette arrangement in the variable region can be altered by excision or reassortment, through the action of the integron integrase (2, 3). Gene cassette reassortment could possibly place certain genes closer to the promoter area, thus enhancing expression and resistance (2). All this data suggests that integrons are extremely pliable genetic structures, able to evolve rapidly in order to ensure the survival of the host bacterial species.

To date the role of single antimicrobials, employed at sub-inhibitory levels (as would occur during inappropriate antibiotic therapy), has not been examined in terms of their possible selective effect upon *bla*$_{GES}$-type genes. This study examined well-characterized bacterial isolates (15, 16) with class 1 integron-located *bla*$_{GES}$-type genes for three possible genetic scenarios: i) major events such as cassette integration into the *attI1* site upstream from *bla*$_{GES}$, ii) mutation of the regulatory environment upstream from *bla*$_{GES}$ and iii) spontaneous point mutations occurring within the respective *bla*$_{GES}$ genes. Despite the continuous exposure of isolates to sub-inhibitory levels of antimicrobials over 120 hours of incubation (the average local antibiotic course duration), none of these three events could be demonstrated. In fact, the DNA regions examined, were indistinguishable from those obtained from non-exposed

isolates in this study. Future research should focus on possible recombinational events occurring away from the *attI* site (4), while the organism is under antibiotic stress. From the data obtained, it thus appears unlikely that short-term exposure to a single antimicrobial agent will select for spontaneous mutations in *bla*$_{GES}$-type genes or their immediate upstream regulatory environments. As suggested by a previous study (18), integrons in bacteria originating from a natural habitat appear to preferentially exist without any antibiotic resistance gene cassettes, in the absence of antibiotic selective pressure. Thus, the precise role of prolonged, multi-antibiotic stress in the nosocomial environment as reported from Japan and Greece (20, 21, 22), on the genetic stability of *bla*$_{GES}$-type genes and the integrons that support them, is however still largely uncertain. Only further research conducted over longer time periods and possibly with various combinations of antimicrobials and drug concentrations, will possibly shed some light on the driving force behind spontaneous mutations observed in these genetic structures.

6.5 References

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