CHAPTER 3: INTEGRONS AND BETA-LACTAMASES – A NOVEL PERSPECTIVE ON RESISTANCE.

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3.1 Introduction.

The horizontal transfer of genetic material within and between microbial genera has been instrumental in the emergence of novel antibiotic resistance traits observed worldwide. Rapid and widespread emergence of resistance and more importantly similar patterns of resistance have been encountered in phylogenetically diverse Gram-negative clinical isolates on an increasing scale (1). Apart from well-known bacterial methods of mutation, antibiotic genes are frequently carried in integron-borne cassettes, which provide an efficient means for capturing and exchanging various resistance genes (2). To date four classes of integrons, each with distinct *int* genes, have been described in Gram-negative bacterial isolates (3, 4, 5), with class 1 integrons being most prevalent in clinical isolates, carrying single or multiple gene cassettes. Integron inserted genes encode for various antibiotic resistance mechanisms, including over 40 distinct genes, conferring resistance towards aminoglycosides, beta-lactams, chloramphenicol, macrolides, sulphonamides, antiseptics and disinfectants (3, 5, 6). Concerning beta-lactamases, integron-borne gene cassettes have been found mainly in *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and various *Enterobacteriaceae* species encompassing Ambler classes A (except TEM and SHV types), B and D beta-lactamase enzymes, giving rise to widespread beta-lactam resistance (7, 8, 9, 10). Only eight discrete families of beta-lactamases have so far been reported from classes 1 and 3 integron structures

originating from geographically distinct locations, with some rare exceptions (Tables 3-1 to 3-3).

Table 3-1: Ambler class A, integron-located beta-lactamases reported from various Gram-negative bacterial species.

Beta-lactamase	Host species	Origin	Reference
VEB-1	*Klebsiella pneumoniae*	Vietnam	9
	Escherichia coli	Vietnam	9
	Pseudomonas aeruginosa	France	12
	Citrobacter freundii	Thailand	13
VEB-1a	*Pseudomonas aeruginosa*	Kuwait	14
VEB-1b	*Pseudomonas aeruginosa*	Kuwait	14
VEB-2	*Pseudomonas aeruginosa*	Thailand	15
GES-1	*Klebsiella pneumoniae*	French Guiana	18
	Pseudomonas aeruginosa	France	56
	*Klebsiella pneumoniae**	Portugal	60
GES-2	*Pseudomonas aeruginosa*	South Africa	10, 11
IBC-1	*Enterobacter cloacae*	Greece	16
IBC-2	*Pseudomonas aeruginosa*	Greece	17
CTX-M-2	*Salmonella enterica*	Argentina	19
	Proteus mirabilis	Argentina	20
CTX-M-9	*Escherichia coli*	Spain	21
PSE-1	*Vibrio cholerae*	Thailand	22

All genes listed were found on Class 1 integron structures with notable exceptions.
* - Class 3 integron location.

Table 3-2: Ambler class B, integron-located beta-lactamases reported from various Gram-negative bacterial species.

Beta-lactamase	Host species	Origin	Reference
IMP-1	*Serratia marcescens**	Japan	23, 24, 25
	Pseudomonas aeruginosa	Japan	44, 45
IMP-2	*Acinetobacter baumannii*	Italy	26
IMP-3	*Shigella flexneri*	Japan	47, 48
IMP-4	*Acinetobacter baumannii*	Hong Kong	8
	Citrobacter youngae	China	49
IMP-6	*Serratia marcescens*	Japan	50
IMP-7	*Pseudomonas aeruginosa*	Canada	7, 51
IMP-8	*Klebsiella pneumoniae*	Taiwan	27
IMP-12	*Pseudomonas putida*	Italy	28
VIM-1	*Acinetobacter baumannii*	Italy	7
	Pseudomonas aeruginosa	Italy	46
	Achromobacter xylosoxydans	Italy	29
	Pseudomonas aeruginosa	Greece	52
VIM-2	*Pseudomonas aeruginosa*	France	53, 54
	Pseudomonas aeruginosa	Italy	55
	Pseudomonas aeruginosa	Spain	56

All genes listed were found on Class 1 integron structures with notable exceptions.

* - Class 3 integron location.

Table 3-3: Ambler class D, class 1 integron-located beta-lactamases reported from various Gram-negative bacterial species.

Beta-lactamase	Host species	Origin	Reference
OXA-1	*Salmonella enterica*	Italy	32
OXA-5	*Pseudomonas aeruginosa*	South Africa	10, 11
OXA-9	*Enterobacter aerogenes*	France	33
OXA-10	*Pseudomonas aeruginosa*	Vietnam	9
OXA-11	*Pseudomonas aeruginosa*	Turkey	31
OXA-14	*Pseudomonas aeruginosa*	Turkey	31
OXA-16	*Pseudomonas aeruginosa*	Turkey	31
OXA-19	*Pseudomonas aeruginosa*	France	30
OXA-20	*Pseudomonas aeruginosa*	France	57
OXA-28	*Pseudomonas aeruginosa*	France	31
OXA-30	*Escherichia coli*	France	59

This apparent restriction of geographical range regarding enzyme families can be ascribed to various factors including genetic incompatibility, geographic location, antibiotic selective pressure and selective research interests (6, 7, 10, 11). Although complex, understanding the underlying genetic mechanisms responsible for the acquisition and spread of these unique beta-lactamase mediated antibiotic resistance mechanisms, could eventually facilitate development of effective prevention and control strategies.

3.2 Epidemiology.

Beta-lactamase VEB-1 was the first class A enzyme found to be encoded by an integron-located gene cassette (9), demonstrating horizontal transfer in the same patient, between different genera belonging to the *Enterobacteriaceae*. The same gene was subsequently found in *P.aeruginosa* and *Escherichia coli* strains in France, originating from patients previously hospitalised in South-East Asia (12). A study conducted in Thailand found *bla*$_{\text{VEB-1}}$ and *bla*$_{\text{VEB-1-like}}$ genes in *P. aeruginosa*, *Citrobacter freundii* and *Pseudomonas putida* (13), with clonal dissemination of *bla*$_{\text{VEB-1,}}$ demonstrated by pulsed field gel electrophoresis (PFGE) in *P. aeruginosa*. Integron-borne VEB-1a and VEB-1b type enzymes were found in *P. aeruginosa* isolates originating from Kuwait (14), while *bla*$_{\text{VEB-2}}$ was found on a class 1 integron structure in *P. aeruginosa* from Thailand (15).

The closely related class A beta-lactamases belonging to the GES / IBC group exhibit a widely scattered distribution with isolates in France, Greece, French Guinea and South Africa (10, 11, 16, 17). GES-1, the first described enzyme from this group was found on a class 1 integron element In52, in a *Klebsiella pneumoniae* isolate originating from French Guinea (18). Virtual simultaneous description of the closely related integron-borne cephalosporinase IBC-1 followed, the *bla*$_{\text{IBC-1}}$ gene originating from an *Enterobacter cloacae* strain found in Greece (16). The simultaneous detection of closely related enzymes from geographically distinct locations most likely signifies the hidden potential of the GES / IBC type enzymes among strains belonging to the *Enterobacteriaceae*. Both GES-1 and IBC-1 were found on highly mobile gene cassettes located in class 1 integron structures, with typical GC content suggestive of an enterobacterial origin (16, 18). Further developments revealed the possible horizontal transfer of *bla*$_{\text{GES}}$ and *bla*$_{\text{IBC}}$ genes from *Enterobacteriaceae* to *P.*

aeruginosa (10, 17), with GC content analysis clearly revealing the incompatibility of *bla*$_{GES-2}$ with *P. aeruginosa* DNA (10). A small outbreak caused by GES-2-producing *P. aeruginosa* in a South African tertiary hospital, revealed clonal dissemination of this resistance threat amongst clinical isolates (11). A recent development in the GES-1 evolutive saga came in the form of *bla*$_{GES-1}$ located on a class 3 integron, isolated from a *K. pneumoniae* isolate in Portugal (60). The clinical significance of this specific genetic localization is however still largely uncertain.

A novel class 1 integron (InS21) carrying the class A *bla*$_{CTX-M-2}$ gene, was found in a *Salmonella enterica* serovar infantis isolate, originating from Argentina (19). The dissemination of *bla*$_{CTX-M-2}$ between bacterial genera via class 1 integron structures is evident from previous findings, locating CTX-M-2 in the variable region of a class 1 integron present in a high molecular weight plasmid originating from a *Morganella morganii* isolate (19). Analysis of a *Proteus mirabilis* strain originating from Argentina revealed *bla*$_{CTX-M-2}$ on an unusual class 1 integron (In35) (20). Sequence analysis of the *bla*$_{CTX-M-2}$ gene and surrounding DNA revealed 99% homology with the chromosomally borne, class A *bla*$_{KLUA-1}$ gene, originating from *Kluyvera ascorbata* (20). Data from that study suggests plasmid acquisition of *bla*$_{CTX-M-2}$ through an uncharacterised recombinational event incorporating open reading frames of unknown function. The latest integron-borne CTX-M-type enzyme was found in Spain, carried on a complex *sul1*-type, class 1 integron originating from *E. coli* (21). This enzyme was subsequently identified as CTX-M-9. Another rare find was made when *bla*$_{PSE-1}$ was discovered on a class 1 integron, originating from *Vibrio cholerae* in Thailand (22).

Regarding beta-lactamases from Ambler class B, to date only two distinct families have made their appearance on integrons. IMP type enzymes found on class 1

integron structures include IMP-1 – 4, IMP-6 – 8 and IMP- 12 (Table 3-2). These integron-borne genes were reported from geographic locations as diverse as Japan, Hong Kong, Taiwan, China, Italy and Canada (Table 3-2). IMP-1 however, made its appearance on a class 3 integron structure, isolated from a high molecular weight transferable plasmid originating from a *Serratia marcescens* isolate in Japan (23, 24, 25). Of interest, the $bla_{\text{IMP-2}}$ gene found in an *A. baumannii* isolate from Italy directed the attention on spontaneous development of this gene in an isolate unrelated to the Far East (26). This finding suggests that the reservoir for IMP-type genes could be widespread and could pose a serious threat for global dissemination. Finally, the isolation of integron-borne $bla_{\text{IMP-8}}$ in *K. pneumoniae* from Taiwan (27) and $bla_{\text{IMP-12}}$ in *P. putida* from Italy (28) respectively, concludes the current integron based evolution of this enzyme family.

The second class B enzyme type found on class 1 integron structures are the enzymes belonging to the VIM family, namely VIM-1 and VIM-2. They were found mainly on integron structures in *A. baumanii* and *P. aeruginosa* isolates, mostly originating from European locations such as Italy, France, Greece and Spain (Table 3-2). Integron-mediated spread to other non-fermenting Gram-negative and *Enterobacteriaceae* species has been documented in Italy and Greece, with $bla_{\text{VIM-1}}$ genes detected in clinical isolates of *Achromobacter xylosoxidans* (29) and *E. coli* respectively (Unpublished data [Genbank accession # AY152821]). Despite their predominant integron genetic support, the VIM-type genes tend to be currently located in the Mediterranean region and France, the exception being however the chromosome located, non-integron mediated, metallo-beta-lactamase gene $bla_{\text{VIM-3}}$ discovered in Taiwan (7).

Class D beta-lactamases found on integron structures, belong to the OXA type family (Table 3-3). They tend to occur mainly on class 1 integrons isolated from *P. aeruginosa* (9, 10, 30, 31), but in isolated instances have been found to occur in integron-borne form isolated from *Salmonella enterica* serotype typhimurium and *Enterobacter aerogenes* respectively (32, 33). OXA-type genes found on class 1 integrons from *Enterobacteriaceae* tend to be carried as the sole *bla*-gene cassette on the integron, in conjunction with numerous other co-resistance genes (32, 33). In contrast, OXA-type integron-borne gene cassettes in *P. aeruginosa* tend to be the secondary *bla*-gene cassette on the integron, with class A-type *bla*-genes mostly functioning as the primary integron-borne beta-lactamase (9, 10, 31).

3.3 Genetic determinants.

Integrons are genetic elements able to capture individual antibiotic resistance genes including those encoding various beta-lactamases and in the process promote their transcription and expression (4, 34, 35). Integrons include a receptor site, *attI*, where captured genes are integrated, together with an adjacent sequence coding for a recombinase, IntI (35). Uncaptured gene cassettes exist in their free form as circular molecules consisting only of one open reading frame (ORF) and a 59-base element (59-be) situated downstream (3, 35, 36). Integration of these gene cassettes involves IntI-catalysed site-specific recombination between the integron associated *attI* site and the 59-be recombination site, associated with the incoming gene cassette (35). Each gene cassette has a unique 59-be, implying that 59-be associated with specific gene cassettes may differ both in sequence and length (35, 37). The 59-be family exhibits a common inverted repeat structure and consensus sequences at each end that consists of approximately 25 bases (35). The outer boundaries of the 59-be in addition contain

the conserved seven base pair core site GTTRRRY at the recombinant cross-over point, and an inverse core site RYYYAAC at the 3' end of the inserted gene cassette (10, 35, 38). Integron cassettes identified to date are in the same orientation, the core site of an integrated cassette is complementary to the inverse core site of the next cassette downstream (6).

Although integrons are considered to be natural expression vectors, great variability of the expression level of integrated gene cassettes are exhibited. This variability is linked to the intrinsic structures of both gene cassettes and integrons (34). Gene expression in an integron is dependant on various factors including promoter strength, gene copy number, the relative distance of the gene cassette from the promoter, and the presence of additional internal promoters (3, 4). Expression is usually mediated via a common promoter situated upstream (5` end) of gene cassettes, rather than through individual promoters. Higher levels of gene expression can be achieved if a second promoter is included adjacent to the first, or if the gene in question is included as multiple copies (3). The relative distance between a gene cassette and the promoter plays a significant role regarding expression, proximal genes tend to be expressed more effectively than distal genes. As a result distal genes may be poorly expressed and have very little effect on the susceptibility of the host bacterium to relevant antibiotics (3, 4).

Integron carriage of resistance gene cassettes by the host bacterium was found to be dependent on the environment that the host organism found itself in, with loss of integron-borne resistance genes in the absence of antibiotic selective pressure (5). Stability of integron gene cassette arrangement and therefore gene expression, during or after an antibiotic onslaught on the organism remains undetermined and may prove

helpful in understanding the role that antibiotics play in the evolution and possible future control of these genetic structures in the nosocomial environment.

3.4 Expression of co-resistance.

The presence of co-resistance gene cassettes (set of resistance genes, conferring resistance to different antimicrobial classes) on integrons, make these genetic elements extremely useful to bacteria, as resistance to a variety of non-related substances can be conferred simultaneously. Additionally, integron-mediated multi-drug resistance tends to favour co-selection of isolates, thereby facilitating widespread dissemination through patients from a wide variety of clinical disciplines (11). Most integron-borne beta-lactamase genes are situated on class 1 integrons, as is evident from the typical genetic structure and the co-resistance genes to quaternary ammonium compounds (*qacΔE1*) and sulphonamides (*sul1*) that classically occur at the distal 3`-end (10, 18, 39). Gene cassettes encoding aminoglycoside resistance tend to co-occur commonly with beta-lactamase gene cassettes on integron structures, with *aac*-type (aminoglycoside acetyltransferase) and *aad*-type (aminoglycoside adenyltransferase) genes occurring most often (Tables 3-4 and 3-5).

Fusion of two aminoglycoside resistance gene cassettes co-occurring with $bla_{\text{GES-1}}$ on a class 1 integron, has been reported from France with fusion of an *aac(3)-I* to an *aac(6`)* cassette (39). In that study it was proved that the fused gene product, as well as the two separate cloned genes conferred resistance to various aminoglycosides. The fused gene product however exhibited a wider resistance repertoire towards aminoglycosides than the two separate genes; this may be an indication towards future development trends in integron-borne aminoglycoside resistance (39).

Table 3-4: Integron-borne co-resistance genes reported to occur with class A, beta-lactamase genes.

Beta-lactamase	Co-resistance genes located on integron 5`-end 3`-end	Host species	Reference
VEB-1	$bla_{\text{OXA-10-like}}$	*C. freundii, P. aeruginosa P. putida*	13
	$bla_{\text{OXA-10-like}}$, *arr*-2-like	*E. coli, Enterobacter spp, K. pneumoniae*	40
	arr-2, *cml*A-5, $bla_{\text{OXA-10-like}}$, *aad*B, *aad*A1	*P. aeruginosa*	15
VEB-2	*arr*-2, *cml*A-5, $bla_{\text{OXA-10-like}}$, *aad*B, *aad*A1	*P. aeruginosa*	15
GES-1	*aac(6`)-Ib`*, *dfr*XVb, *cml*A4, *aad*A2, *qac*EΔ1, *sul1*	*K. pneumoniae*	18
	*aac(3)-Ib / aac(6`)-Ib`, qac*EΔ1, *sul1*	*P. aeruginosa*	39
	bla_{OXA}, *aac(6`)-Ib*	*K. pneumoniae*	60
GES-2	$bla_{\text{OXA-5}}$, *aac(3)-I, qac*EΔ1, *sul1*	*P. aeruginosa*	10, 11
IBC-1	*aac(6`)-Ib, qac*EΔ1, *sul1*	*Enterobacter cloacae*	16
IBC-2	None found.	*P. aeruginosa*	17
CTX-M-2	*aac(6`)-Ib*, $bla_{\text{OXA-2}}$, *qac*EΔ1, *sul1*	*Salmonella enterica* serovar infantis	19
	aac(6`)-Ib, $bla_{\text{OXA-2}}$, *qac*EΔ1, *sul1*	*Proteus mirabilis*	20
CTX-M-9	*dfr*A16, *aad*A2, *qac*EΔ1, *sul1*	*Escherichia coli*	21
PSE-1	*aad*B, *qac*EΔ1, *sul1*	*Vibrio cholerae*	22

Table 3-5: Integron-borne co-resistance genes reported to occur with class B, beta-lactamases.

Beta-lactamase	Co-resistance genes located on integron 5`-end 3`end	Host species	Reference
IMP-1	*aac(6`)-Ib, bla*$_{TEM-1}$ *aacA4, catB6, qacG, qacEΔ1, sul1* *aac(6`)-Ib*	*Serratia marcescens* *P. aeruginosa* *P. aeruginosa*	* 7 44
IMP-2	*aacA4, aadA1*	*Acinetobacter baumannii*	26
IMP-3	No data available		
IMP-4	*qacG, aacA4, catB3*	*Acinetobacter* spp.	8
IMP-6	No data available		
IMP-7	*aacC4, aacC1*	*P. aeruginosa*	51
IMP-8	*aac(6`)-Ib, catB4, qacEΔ1, sul1.*	*Klebsiella pneumoniae*	27
VIM-1	*aacA4, aphA15, aadA1, qacEΔ1, sul1* *aacA4*	*Achromobacter xylosoxidans* *P. aeruginosa*	29 46
VIM-2	*qacEΔ1, sul1* *aacA7, aacC1, qacEΔ1, sul1* *aacA29a, aacA29b, qacEΔ1, sul1* *aacA4, qacEΔ1, sul1* *aac(3)-Ic, cmlA7*	*P. aeruginosa* *P. aeruginosa* *P. aeruginosa* *P. aeruginosa* *P. aeruginosa*	53 7, 54 7, 54 55 58

* Unpublished data (Genbank Accession # AB070224)

Sequential trimethoprim and sulphonamide resistance is in some cases encoded for on two different gene cassettes within the same integron; the *sul*-type genes

(dihydropterorate synthase) and *dhfr* / *dfr*-type genes (dihydrofolate reductase) commonly occur together on class 1 integrons in conjunction with class A *bla* gene cassettes (Table 3-4). *Sul*-type gene cassettes commonly occur in the 3`-conserved regions of class 1 integrons (10). In most cases the *dhfr / dfr* – type gene cassettes are situated closer to the promoter region (5`- end) of the integron and should therefore be expressed more efficiently than the *sul* – type cassettes (Table 3-4). The clinical significance of this phenomenon is however still uncertain. Resistance towards rifampin encoded by the *arr*-type genes (rifampin ADP-ribosylating transferase) was only found in isolated cases, co-occurring on class 1 integrons, with $bla_{\text{VEB-1}}$ and $bla_{\text{VEB-2}}$ genes originating from Thailand and Vietnam respectively (15, 40). Two different integron-borne genes conferring resistance towards chloramphenicol have been described to date. Gene cassettes of the *cml*–type (chloramphenicol resistance protein) and *cat*–type (chloramphenicol acetyltransferase) were found to co-occur on class 1 integrons with class A and class B beta-lactamase gene cassettes, respectively (8, 15, 18, 27).

Considering beta-lactamase genes as co-resistance factors co-occurring with primary *bla* gene cassettes on integrons, mainly the class D oxacillinases or bla_{OXA}-type genes have been reported to occur significantly on both class 1 and 3 integrons (Table 3-4). These gene cassettes are most frequently reported from integrons originating from non-fermenting bacterial species such as *P. aeruginosa* (10, 15), and rarely made an appearance as secondary beta-lactamase gene cassettes on integrons originating from *Enterobacteriaceae* (13, 19, 20). Although none of the class A or D type beta-lactamase genes have been described previously to co-occur together with integron-borne class B type genes, a rare exception was found in the form of $bla_{\text{TEM-1}}$, co-occurring with $bla_{\text{IMP-1}}$ originating from a *S. marcescens* clinical isolate (Unpublished

data. [Genbank accession # AB070224]). To date, this is the only description of an integron-borne *bla*$_{TEM}$ gene. Secondary beta-lactamase gene cassettes are most commonly recruited into integron structures to fill very specific niches in the organism's resistance profile against beta-lactam antibiotics (10, 11), possibly reflecting on regional selective pressures.

3.5 Detection.

Detection of integron-borne resistance gene cassettes has traditionally been relegated to dedicated research institutions rather than the clinical microbiology laboratory. The most likely explanation for this can mostly be related to the costs, personnel time involved and slow turn around time of the diagnostic process. However, even with modern molecular techniques, the specific identification of all the cassettes inserted into an integron structure may take from several days to weeks or longer, to unravel. This slow identification process is clearly of very little clinical benefit to individual patients in the ICU. Developments in the field of real-time PCR detection of class 1 integrons (41) from *Enterobacteriaceae* and non-fermenting Gram-negative bacteria may prove to revolutionise this particular field of research. Utilisation of a primer pair specific for a 300-base pair (bp) conserved region at the 5`-end of class 1 integrons, was shown to be both highly sensitive and specific to detect these genetic structures from various bacterial species (41). That study utilised the LightCycler instrument and results obtained, compared favourable with conventional PCR detection of class 1 integrons (41). Primers designed for the 5'- and 3'- conserved ends of class 1 integrons may prove to be a valuable screening tool to further detect beta-lactamase and other co-resistance genes that form part of class 1 integron structures (42, 43),

especially when used in conjunction with the phenotypic exhibition of co-resistance factors revealing the likely presence of integron-mediated resistance.

When an integron is detected, standard PCR conditions with a series of primers designed for detection of the more prevalent classes A and B, beta-lactamase genes, such as bla_{VEB}, $bla_{GES/IBC}$, bla_{IMP}, and bla_{VIM} could be used to identify specific genes (9, 18, 44, 45, 46, 61). A thorough knowledge of circulating local strains and resistance patterns (the place to place phenomenon) are extremely helpful in targeting a molecular approach in order to conduct cost-effective screening. Sequencing of PCR products is unfortunately still the preferred method of differentiation between beta-lactamase genes and could substantially add to the cost of a screening program, a factor most developing countries can hardly afford. Future research should focus on quick, simplified and cost effective molecular methods that could bring integron identification into the realm of the clinical microbiology laboratory.

3.6 Conclusion.

Reports of structurally related, integron-located ESBL and carbapenemase genes in mainly *P. aeruginosa, A.baumannii* and the *Enterobacteriaceae* from different parts of the world, paint a bleak picture in terms of evolutive transfer of beta-lactamase-mediated antibiotic resistance, with questions arising concerning the origin of these genes. Further research gaining knowledge about integron-related antibiotic selective pressure, is absolutely crucial in order to stem the tide of bacterial resistance in the nosocomial environment.

3.7 References.

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