CHAPTER 2: AMBLER CLASS A EXTENDED-SPECTRUM BETA-LACTAMASES IN *PSEUDOMONAS AERUGINOSA* – NOVEL DEVELOPMENTS AND CLINICAL IMPACT.

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2.1 Introduction and epidemiology.

The so-called clavulanic-acid inhibited extended-spectrum beta-lactamases (ESBLs) belong mostly to class A of the Ambler classification scheme (1) and confer resistance to at least several expanded-spectrum cephalosporins (21, 22, 34) (Table 1-1, 1.1). They have been extensively reported in *Enterobacteriaceae* from the early 1980`s whereas they have been described in *Pseudomonas aeruginosa* only more recently (22, 34, 40). These enzymes described in *P. aeruginosa*, are either of the TEM- and SHV-types that are also well known in *Enterobacteriaceae*, of the PER-type mostly originating from Turkish isolates, or of the VEB-type from Southeast Asia and, more recently, of the GES /IBC types reported from France, Greece and South Africa, respectively (14, 33, 37-39, 41, 48, 52). These five types of enzymes are remotely related, both from a genetic point of view and similarities in hydrolytic profiles. Recent studies indicated that these enzymes may play an important role in the dissemination of antibiotic resistant isolates and may condition future choices of antibiotic regimens for treating life-threatening infections due to ESBL-producing *P. aeruginosa* (12, 17, 51).

As summarized in Table 2-1, these enzymes have been found so far in a limited number of geographical areas, suggesting that some of these beta-lactamase genes may at least in several cases represent a specific local selection.

Table 2-1. Ambler Class A extended-spectrum beta-lactamases occurring in *P. aeruginosa.*

Beta-lactamase	Genetic support	Country of first isolation	Year of first isolation	Other countries of isolation	Reference
VEB-1	C/P/I	France	1998	Thailand, India,* China*	6, 18, 38, 60
VEB-1a	C/I	Kuwait	1999	-	50
VEB-1b	C/I	Kuwait	1999	-	50
VEB-2	C/I	Thailand	1999		18
PER-1	C	France	1991	Turkey, Italy, Belgium	8, 12, 30, 39, 41, 61, 63
SHV-2a	C/P	France	1995	Thailand, Poland*	37
SHV-5	P	Thailand	1994-1996	Greece *	6
SHV-12	C	Thailand	1994-1996	-	6
TEM-4	P/C	France	1996	-	42
TEM-21	C	France	1997	-	13
TEM-24	P	France	1998	-	31
TEM-42	P	France	1992	-	36
GES-1	C/I	France	1999	-	14
GES-2	P/I	South Africa	2000	_	51, 52
IBC-2	C/I	Greece	1998	_	33

C, chromosomal location; P, plasmid borne; I, integron-borne.
* P. Nordmann, personal communication.

The SHV-type ESBLs have been identified in very rare isolates of *P. aeruginosa*, SHV-2a from France, whereas SHV-5 and SHV-12 were from Thailand (6, 37). These isolates were nosocomial strains except the SHV-12 producer, isolated from a clinical sample from an outpatient of a Thai hospital (6).

The TEM enzymes described in *P. aeruginosa* namely, TEM-4, TEM-21, TEM-24 and TEM-42 have been reported in rare isolates from France (3, 13, 31, 36, 42, 49). A French survey indicated that only 10% of ticarcillin-resistant *P. aeruginosa* produce a TEM-type beta-lactamase, whereas other narrow-spectrum beta-lactamases (OXA, CARB) constitute a higher proportion in that species (3). Conversely, the TEM-type

enzymes are widely distributed in *Enterobacteriaceae*, whereas OXA-type and CARB-type beta-lactamases are rare (27). The few reports of *P. aeruginosa* strains harbouring TEM and SHV-type genes may have several explanations. Firstly, the rarity of narrow-spectrum TEM and SHV-type enzymes may limit antibiotic selection of these enzymes with an expanded-spectrum hydrolysis. Secondly, a higher proportion of acquired oxacillinase and carbenicillinase genes (most of them being chromosome-encoded) may fulfil the function of genes encoding narrow-spectrum enzymes such as the TEM and SHV types. Indeed, several oxacillinases (OXA-2 and OXA-10 derivatives, and OXA-18) have been reported in *P. aeruginosa* that have extended substrate profiles including extended-spectrum cephalosporins (7, 40, 45). Thirdly, expression of the chromosome-encoded cephalosporinase of *P. aeruginosa* may be up regulated (derepression) and may thereby be a convenient way for acquisition of resistance to expanded-spectrum cephalosporins (23), without the need for expanding its genetic repertoire. It is likely that the origin of TEM- and SHV-type ESBLs in *P. aeruginosa* may result from gene transfer from *Enterobacteriaceae* (27). This has been shown for TEM-24 (31) and the downstream-located DNA sequences of the chromosome of *P. aeruginosa* RP-1 that produces SHV-2a, which were found to be identical to those reported as plasmid-encoded in a *Klebsiella pneumoniae* isolate (37, 46). Differences in replication origins of plasmids from *Enterobacteriaceae* and *P. aeruginosa* may however limit such intergeneric transfers. Additionally, difficulty of detection of TEM- and SHV-type ESBLs in the clinical laboratory may underestimate their true prevalence in *P. aeruginosa*.

Beta-lactamase PER-1 was the first ESBL identified and fully characterized in *P. aeruginosa* in 1993 (39, 41). It was found from a *P. aeruginosa* isolate of a Turkish patient hospitalised in the Paris area in 1991 (39). A subsequent study on the

distribution of the *bla*$_{PER-1}$ gene revealed that it is widespread in Turkey, with PER-1 being identified in up to 46% of *Acinetobacter* strains and 11% of *P. aeruginosa* analysed in a nation-based survey performed over a three-month period in 1999 (61). PER-1 was identified in up to 38% of ceftazidime-resistant *P. aeruginosa* isolates, with ribotyping results indicating spread of different clones (61). Since screening for the *bla*$_{PER-1}$ gene has not been performed in *P. aeruginosa* isolates originating from countries located to the south and east of Turkey such as Syria, Iran and Iraq, no current data are available on the prevalence of PER-1 in the Middle East.

It is possible that the spread of PER-1 in Western Europe may be mostly related to the widespread immigration of Turkish nationals. Interestingly, although reported in several enterobacterial species including community-acquired pathogens such as *Salmonella* spp. (62), beta-lactamase PER-1 seems mostly expressed from *P. aeruginosa* and *Acinetobacter* spp. isolates in Turkey (61, 63). A large nosocomial outbreak of PER-1-producing *P. aeruginosa* has been documented in Varese, Italy, occurring over a 10-month period in a tertiary hospital (30). During that outbreak, a total of 108 clinical isolates were recovered from 18 patients, reflecting the propensity of *P. aeruginosa* to widely colonize hospitalised patients. In that case, apart from the beta-lactam resistance phenotype conferred by PER-1, epidemic strains were resistant to several disinfectants, including chlorhexidine, iodide povidone, and toluene-*p*-sulphochloramide (30). Control of the outbreak was achieved by implementing strict hygienic measures, carbapenem therapy and disinfection of decubitus ulcers and surgical wounds with mercurochrome or silver nitrate solutions (30). As a result of increased carbapenem consumption, selection of several carbapenem-resistant organisms occurred in the nosocomial environment including OprD-defective *P. aeruginosa*, *Stenotrophomonas maltophilia* and *Pseudomonas putida* producing the

class B carbapenemase VIM-1 (30). The same group had reported a *P. aeruginosa* strain that produced the plasmid-mediated beta-lactamase VIM-2 together with beta-lactamase PER-1 (12) thus showing that the same *P. aeruginosa* strain may produce two unrelated beta-lactamases both with expanded-spectrum hydrolysis. Recently, another *P. aeruginosa* strain that produced PER-1 has been isolated from a patient hospitalised in Clermont-Ferrand in the central part of France (11). Indeed, this latter patient had been hospitalised previously in Strasbourg, in the eastern part of France where the patient might have been in contact with hospitalised Turkish patients (P. Nordmann, personal communication). A pseudo-outbreak (false positive culture results due to specimen contamination) has also been reported from Belgium (8), revealing the obstacles that face investigators when searching for the source of multiresistant *P. aeruginosa* isolates. Although no mention is made about the antibiotic regimen used for treating the infected patient, the pseudo-outbreak was successfully terminated by decontamination of a side-room urine densitometer (8).

Another unrelated ESBL from *P. aeruginosa*, i. e. beta-lactamase VEB-1 was originally identified in *E. coli* and *Klebsiella* isolates from a 4-month old Vietnamese child transferred from Vietnam and hospitalized in France (48). Subsequent isolation of VEB-1 from *P. aeruginosa* was documented from two patients hospitalised in France and transferred from Thailand (38). A study conducted in a university hospital in Thailand (17), revealed that *bla*$_{VEB}$-like genes were present in up to 93% of the ceftazidime-resistant isolates whereas ceftazidime resistance occurred in 24% of *P. aeruginosa* isolates. As this is an isolated study, the clinical significance of VEB-type enzymes in the region is still uncertain. Another *bla*$_{VEB-1}$-like gene, *bla*$_{VEB-2}$ had been identified during this study, with VEB-2 differing from VEB-1 by only one amino acid change located outside the active site of the enzyme (17). The latest development in

analysis of *bla*$_{VEB-1}$-like genes was the isolation of *P. aeruginosa* strains from an intensive care unit of a Kuwaiti hospital harboring *bla*$_{VEB}$–like genes, *bla*$_{VEB-1a}$ and *bla*$_{VEB-1b}$, that differ from the *bla*$_{VEB-1}$ gene by nucleotide substitutions in the DNA sequence encoding the leader peptide (50) (Table 2-1). Unpublished data have also identified VEB-1 from *P. aeruginosa* in India and China (P. Nordmann, personal communication). It is likely that VEB-type enzymes may be isolated mostly from patients coming from or hospitalised in Southeast Asia.

Another ESBL, beta-lactamase GES-1, was first identified from a French Guiana *K. pneumoniae* strain isolated in Paris (47). Subsequently, *bla*$_{GES-1}$ was identified from a *P. aeruginosa* isolate in France (14) as well as the structurally related *bla*$_{IBC-2}$ gene from a Greek isolate in Thessaloniki (33). These results suggested that these ESBL genes might have a wider scattered distribution than VEB and PER enzymes. One of the most interesting developments in research in ESBLs in *P. aeruginosa* is the identification of GES-2 that differs from GES-1 by a single amino acid change located in the active site of these enzymes (52). GES-2 hydrolyses not only extended-spectrum cephalosporins but also imipenem to a minor extent. This enzyme was identified in *P. aeruginosa* from a university hospital in Pretoria, South Africa, and was associated with an outbreak occurring in the same hospital from March to July 2000 (51). Seventy-two ceftazidime-resistant isolates were isolated from nine patients, whereas only eight of these patients were found to harbour seventy clonally related isolates carrying the same *bla*$_{GES-2}$ gene. Presence of the *bla*$_{GES/IBC}$ genes in *P. aeruginosa* and in other gram-negative rods in different countries might indicate a yet undiscovered potential spread of these enzymes.

The only clavulanic-acid inhibited oxacillinase from *P. aeruginosa* with extended-spectrum profile is OXA-18, that has been reported only once from a *P. aeruginosa* strain isolated in Paris, from a patient previously hospitalized in Italy (45). Another

putative but not yet fully characterised ESBL identified in Tunisia (2, 54), again focuses attention on Mediterranean countries as possible reservoirs of ESBL-producing *P. aeruginosa* isolates. Additionally, other non-characterized ESBLs have been described from *P. aeruginosa* isolates in Brazil (44) and in Poland (64).

2.2 Substrate profile.

Concerning the hydrolytic properties of TEM-type ESBLs found in *P. aeruginosa* (13, 31, 36), they are similar to those of classical TEM-type ESBLs hydrolyzing narrow-spectrum penicillins, extended-spectrum cephalosporins and the monobactam aztreonam (4, 34). TEM-4 has a substrate profile that includes mostly cefotaxime (Table 2-2), whereas TEM-42 exhibits a low K_m value for ceftazidime (36), which is widely regarded as an excellent indicator for ESBL activity. The high relative rate of hydrolysis (V_{max}) measured against several extended-spectrum cephalosporins and the overproduction of the TEM enzymes in *P. aeruginosa* may make these enzymes powerful tools for beta-lactam resistance in *P. aeruginosa* (36).

Beta-lactamase SHV-2a mirrors the affinities of TEM-4 to some extent (Table 2-2) with high affinity for latest developed cephalosporins such as cefpirome (37, 46). The kinetic constants of SHV-5 reveal a subtle difference in substrate profile when compared to that of the TEM enzymes, most notably that of TEM-42 (36). Although rare in *P. aeruginosa* and only described in France and Greece (P. Nordmann, personal communication), SHV-5 tends to hydrolyse ceftazidime and cephaloridine most efficiently. Other less favourable substrates include aztreonam, the narrow-spectrum cephalosporins and other extended-spectrum cephalosporins (40). SHV-12 confers resistance towards the extended-spectrum cephalosporins and aztreonam with a marked inhibitory effect of clavulanic acid (6).

Table 2-2. Comparative kinetic parameters for extended-spectrum beta-lactamases found in *P. aeruginosa* [a]

Antibiotics	Kinetic parameters																	
	GES-1			GES-2			PER-1			VEB-1			TEM-4			SHV-2a		
	kcat	*Km*	*Vmax / Km*	*kcat*	*Km*	*Vmax / Km*	Vmax	*Km*	*Vmax / Km*	Vmax	*Km*	*Vmax / Km*	Vmax	*Km*	*Vmax / Km*	Vmax	*Km*	*Vmax / Km*
Benzylpenicillin	2.8	40	70	0.4	4	96	100	2.5	40	100	2.8	100	NA	NA	NA	33	17	1.9
Amoxicillin	13	200	65	0.7	25.8	26	174	54	3	110	6.0	50	50	NA	NA	NA	NA	NA
Ampicillin	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	47	28	1.7
Ticarcillin	0.3	400	0.7	0.06	13.3	4.5	7	1.7	4	8	1	22	NA	NA	NA	NA	NA	NA
Piperacillin	8	900	13	0.3	22.8	23	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Cephalothin	179	3400	52	0.3	3	112	473	7	68	700	6.0	325	NA	NA	NA	NA	NA	NA
Cephaloridin	53	2000	26	0.5	7.7	65	356	16	22	2300	12	533	232	NA	NA	100	30	3.2
Cefoxitin	0.9	30	33	NH	NH	NH	< 0.5	0.04	NA	NA	NA	NA	< 1	NA	NA	NA	NA	NA
Cefuroxime	NA	NA	NA	NA	NA	NA	668	18	37	2000	24	230	NA	NA	NA	NA	NA	NA
Ceftazidime	380	2000	188	ND	> 3000	ND	2470	148	17	NA	NA	NA	10	NA	NA	NA	NA	NA
Cefotaxime	68	4600	15	2.2	890	2.5	1510	26	58	4300	38	314	300	NA	NA	7	26	0.2
Cefepime	2.8	1800	1.6	1.1	1900	0.6	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Cefpirome	NA	NA	NA	NA	NA	NA	772	35	22	NA	NA	NA	NA	NA	NA	20	151	0.14
Imipenem	0.003	45	0.07	0.004	0.45	9	< 0.5	0.07	NA	NA	NA	NA	< 1	NA	NA	NA	NA	NA
Meropenem	NH	NH	NH	NH	NH	NH	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Aztreonam	NH	NH	NH	NH	NH	NH	1	23	0.04	400	500	2	< 1	NA	NA	NA	NA	NA

Data were adapted from references 39, 42, 46-48, and 52.
[a] *kcat* (s^{-1}) [only available for GES-1 and GES-2]; *Km* (μM); *kcat* / *Km* ratio ($mM^{-1} . s^{-1}$)
NA – Data not available
NH – Not hydrolyzed (Initial rate of hydrolysis reported lower than 0.001 $\mu M^{-1}. s^{-1}$)
ND – Not determinable due to high *Km* values.

Table 2-3: MICs (µg/ml) of beta-lactams for several representative non-isogenic class A extended-spectrum beta-lactamase producing *P. aeruginosa* isolates.

	Extended-spectrum beta-lactamase producers [b]						
Beta-lactams [a]	**VEB1 (JES-1)**	**PER-1 (RNL-1)**	**GES-1 (695)**	**GES-2 (GW-1)**	**SHV2a (RP-1)**	**TEM-4 (Stel)**	**IBC-2 (555)**
Amoxicillin	> 512	> 512	> 512	> 512	>512	>512	NA
Amoxicillin +CLA	>512	> 512	64	> 512	>512	>512	NA
Ticarcillin	> 512	512	> 512	> 512	>512	>512	> 256
Ticarcillin + CLA	256	256	64	> 512	64	32	> 256
Piperacillin	128	32	512	128	256	32	> 256
Piperacillin + TZB	NA	NA	64	128	16	8	> 256
Cephalothin	>512	> 512	64	> 512	>512	>512	NA
Cefoxitin	NA	> 512	512	> 512	>512	>512	> 256
Ceftazidime	512	128	32	32	32	8	> 256
Ceftazidime + CLA	128	4	32	16	8	4	NA
Cefotaxime	NA	64	NA	128	>512	128	> 256
Cefepime	128	NA	16	32	NA	8	NA
Imipenem	32	0.5	1	16	2	4	> 128
Meropenem	8	NA	NA	16	NA	1	> 128
Aztreonam	>256	256	4	16	32	16	32

[a] CLA, clavulanic acid concentration fixed at 2 µg/ml; TZB, tazobactam concentration fixed at 4 µg/ml.
[b] Well-characterised strain designations appear in brackets.
NA – data not available.
Data were adapted from references 14, 33, 37-39, 49, and 52.

The non-TEM, non-SHV ESBLs from *P. aeruginosa* also tend to exhibit a fairly broad range of substrate specificities (Tables 2-2, 2-3). VEB-1 and PER-1 exhibit the typical substrate profile of classical ESBLs, i.e. high affinities for narrow-spectrum penicillins, narrow- and expanded-spectrum cephalosporins (Table 2-2). PER-1 in particular, exhibits high affinity towards ceftazidime and aztreonam (39), whereas VEB-1 hydrolyses cefotaxime more efficiently than ceftazidime (48). These ESBLs have low-level affinities for the carbapenems and are moderately inhibited by clavulanic acid and imipenem. Beta-lactamases VEB-1a and VEB-1b have the same substrate specificities as VEB-1 since the distinctive mutations were located in the mature protein sequences

outside the putative active site (50). In addition VEB-1 and PER-1 are well inhibited by cefoxitin.

Beta-lactamase GES-1 exhibits a low catalytic activity and low affinity for most substrates (47) and an inhibition profile that includes clavulanic acid and imipenem (Table 2-3). The more recent addition in the GES lineage (GES-2), tends to swing its substrate affinities towards the narrow-spectrum penicillins and carbapenems (52), noticeably against imipenem (Table 2-3). GES-2 has higher affinity for imipenem compared to that of GES-1 (Table 2-3) (52). Although the rate of hydrolysis of imipenem by GES-2 is marginal as compared to that of class B enzymes, GES-2 may confer resistance towards imipenem, most likely when associated with a membrane impermeability or efflux mediated resistance mechanism (52). Inhibition studies of GES-2 revealed a marked increase in its IC_{50} value for imipenem compared to GES-1 (8 ± 2 µM and 0.1 µM, respectively) (52). The IC_{50} value of GES-1 for clavulanic acid compared to that of GES-2 reveals a *ca.* 10^3 factor difference (15 nM and 1 ± 0.5 µM, respectively) (52), that may indicate future selection of a bla_{GES} derivatives with resistance to enzyme inhibitors.

The latest characterized non-TEM non-SHV ESBL in *P. aeruginosa* is IBC-2, reported from a Greek isolate (33). IBC-2 confers resistance to ceftazidime and to other oxyimino-cephalosporins and is inhibited by imipenem, tazobactam and clavulanic acid (33). IBC-2 differs from IBC-1 by one amino acid change occurring outside of the omega loop in Ambler position 104 (Glu to Lys substitution) (16, 33), both enzymes being highly related to the GES-1/GES-2 enzymes (33).

The ability of any beta-lactamase to confer resistance in Gram-negative rods depends on a complex interplay of diffusion of beta-lactam molecules in the periplasmic space, hydrolysis parameters, and produced quantity of the enzyme (gene copy number and

promoter strength) (27). The MIC of a beta-lactam is raised in case of rapid hydrolysis (high *Vmax* or high *kcat*) when the beta-lactamase has high affinity for the substrate (low *Km*) and/or when the diffusion coefficient of the beta-lactam molecule is low (27). Indeed, MICs of beta-lactams for several ESBL producers mirror combinations of these parameters (Table 2-3). In this regard, the permeability diffusion coefficient is especially important for *P. aeruginosa,* in as much that it is a hundred times lower than that of *E. coli* (40). It must be noted however that unlike for *E. coli*, a simple permeability co-efficient for *P. aeruginosa* cannot be calculated (29). When this very low diffusion coefficient is coupled with any other membrane permeability or efflux-related resistance mechanism, the organism may be well protected against broad-spectrum beta-lactams by relatively restricted-spectrum beta-lactamases. This phenomenon is well illustrated by the protection afforded to *P. aeruginosa* against imipenem by beta-lactamase GES-2, whereas the same enzyme fails to induce resistance to imipenem in an *E. coli* clone (51, 52).

Beta-lactamase OXA-18 is the only oxacillinase that is very well inhibited by clavulanic acid (45). Its hydrolytic profile includes extended-spectrum cephalosporins as well as aztreonam. Compared to other OXA-type enzymes in *P. aeruginosa*, its kinetic parameters include both low affinity and high catalytic activity against several cephalosporins. Additionally, like GES-1, its activity is strongly inhibited by imipenem (45).

In addition to acquired beta-lactamases, *P. aeruginosa* has an inducible class C cephalosporinase (AmpC) that confers low-level resistance towards aminopenicillins, narrow-spectrum cephalosporins and cephamycins such as cephalothin and cefoxitin respectively (4, 23). In addition to resistance mediated by efflux and impermeability, resistance to extended-spectrum cephalosporins may arise from i) derepression of

biosynthesis of the chromosomal cephalosporinase (23), ii) acquired secondary beta-lactamases or iii) both mechanisms in tandem (7), making detection of ESBLs very difficult in this species.

2.3 Genetic determinants

The genes encoding the TEM and SHV-type enzymes are usually plasmid-located in *Enterobacteriaceae* (27). Spread of these plasmids may be limited by species-related plasmid replication. Plasmid location of genes encoding ESBLs of the TEM and SHV series has been reported for *bla*$_{\text{SHV-12}}$, *bla*$_{\text{TEM-24}}$, and *bla*$_{\text{TEM-42}}$ in *P. aeruginosa* and simultaneously in enterobacterial isolates from the same patients (6, 31, 36). Whereas *bla*$_{\text{VEB-like}}$ genes are mostly plasmid-encoded in *Enterobacteriaceae*, they are mostly chromosome-encoded in *P. aeruginosa* (17, 18). The same is true for the *bla*$_{\text{PER}}$ -like genes (61-63) whereas the *bla*$_{\text{GES}}$ genes have been found to be either plasmid- or chromosome-encoded in *P. aeruginosa* (14, 33, 51). However, in the latter case, epidemiological surveys are not yet available.

Along with a plasmid location, many antibiotic resistance genes have been identified as a form of gene cassettes and as part of class 1 integrons in *P. aeruginosa*. These genetic structures are vectors of co-localization and of co-expression of antibiotic resistance genes (9, 19, 56). They are associated with typical sequences (59-bp elements) recognized as target sites by the integrase, and that are essential for their mobility (20, 57, 58). The 5' conserved segment of integrons contains the integrase gene (*intI*) and the recombination site *attI1* (53) whereas the 3' conserved sequence carries the antiseptic-resistance determinant *qacEΔ1*, and the *sul1* gene that confers resistance to sulfonamides (25, 43). Whereas genes encoding beta-lactamases of class B (metallo-enzymes) and of class D (oxacillinases) are usually located in class 1 integrons, genes

encoding VEB- and GES-type enzymes are the only genes encoding class A ESBLs that are associated with these genetic structures. Conversely, the *bla*$_{PER-1}$ gene is not integron-associated (40, 41). In several cases, *bla*$_{GES}$ and *bla*$_{VEB}$ genes have been associated in integrons with other beta-lactamase genes *bla*$_{OXA-5}$ and *bla*$_{OXA-10,}$ respectively (18, 51). The gene-associated sequences are almost identical for the *bla*$_{GES}$ genes (the same for the *bla*$_{VEB}$ genes) thus underlining in those cases, epidemic spread of gene cassettes.

Since several integrons have been reported as transposon-located, these structures may provide an additional means of mobility for these antibiotic resistance genes and may explain plasmid and chromosomal locations of the same ESBL gene in *P. aeruginosa* (14, 17, 50-52). Future work should be directed to the identification of the transposon structures that contain these integrons in *P. aeruginosa*. The *bla*$_{VEB-1}$ gene has been identified in *E. coli* within a composite transposon (17, 18) whereas known class 1 integrons are located onto Tn*21* derivatives that are transposons commonly found in *Pseudomonas* spp. (26). Since *bla*$_{VEB}$-like and *bla*$_{GES}$-like genes are integron-located, it is possible that their presence in *P. aeruginosa* may result from horizontal transfer from Gram-negative aerobes (other than *Enterobacteriaceae*) that are known as a source of integrons (56) and that may be present in the same ecological niche.

Recently, the *bla*$_{TEM-21}$ gene was identified as part of a chromosome-located Tn*801* transposon disrupted by insertion of an IS*6100* element (13). The *bla*$_{OXA-18}$ gene was found to be chromosome-encoded, and surprisingly for an oxacillinase gene not integron-associated (45).

2.4 Current detection methods.

The presence of ESBLs in *P. aeruginosa* may be suspected in the face of an antibiotic resistance phenotype combining resistance to ticarcillin and ceftazidime and susceptibility to ticarcillin plus clavulanic acid. Detection of ESBLs using double-disk synergy tests with clavulanate and any extended-spectrum cephalosporins are sensitive and specific in *Enterobacteriaceae* (5, 15, 28). However, the same test may not be as useful for detection of ESBLs in *P. aeruginosa* (8, 40). These difficulties stem from several factors: i) false negative results due to naturally-occurring beta-lactamases such as the chromosome-encoded AmpCs that may be overexpressed ii) simultaneous presence of metallo-enzymes with carbapenem-hydrolyzing activity (IMP and VIM series [12, 32]) or with extended-spectrum oxacillinases (OXA-2 and OXA-10 derivatives) (17), iii) relative resistance to inhibition by clavulanate as examplified by GES-2 (52), and iv) combined mechanisms of resistance such as impermeability and efflux.

Experience indicates that positive results with the double disk synergy test are quite easily obtained with VEB-1- and PER-1-positive strains whereas synergy patterns may be more difficult to detect with GES-type enzymes. In several cases, the synergy image with TEM- and SHV-type ESBLs may be hardly visible in *P. aeruginosa*. Synergy between imipenem and ceftazidime may be observed with *bla*$_{GES}$-like and *bla*$_{PER-1}$ enzymes (14). This synergy may be obscured in some cases by the induction effect of imipenem on the expression of the chromosomal cephalosporinase, exhibiting a concomitant antagonism line between ceftazidime- and imipenem-containing disks. This effect can be overcome to some extent by performing the double-disk synergy test with oxacillin-containing agar plates, since oxacillin inhibits the activity of Ambler class C enzymes (4). The antibiotic resistance phenotype induced by OXA-18 is

identical to that reported for class A clavulanic-acid inhibited ESBLs, making its phenotypic-based differentiation impossible (45).

When an ESBL is suspected in *P. aeruginosa*, PCR-based molecular techniques may help to identify the gene. Standard PCR conditions with a series of primers designed for detection of class A beta-lactamase genes bla_{TEM}, bla_{SHV}, bla_{PER}, bla_{VEB}, and $bla_{GES/IBC}$ could be used (Table 2-4).

Table 2-4. Oligonucleotide primers used for detection of genes encoding class A ESBLs in *P. aeruginosa.*

Primer names	Sequence 5' to 3'	Detection of gene	Reference
VEB-1A	CGACTTCCATTTCCCGATGC	bla_{VEB}	48, 51
VEB-1B	GGACTCTGCAACAAATACGC		
PER-A	ATGAATGTCATTATAAAAGC	bla_{PER}	8, 51
PER-B	AATTTGGGCTTAGGGCAGAA		
GES-1A	ATGCGCTTCATTCACGCAC	$bla_{GES\ /\ IBC}$	51
GES-1B	CTATTTGTCCGTGCTCAGG		
TEM-A	GAGTATTCAACATTTCCGTGTC	bla_{TEM}	51
TEM-B	TAATCAGTGAGGCACCTATCTC		
SWSHV-A	AAGATCCACTATCGCCAGCAG	bla_{SHV}	37
SWSHV-B	ATTCAGTTCCGTTTCCCAGCGG		

However, PCR experiments without further sequencing of the PCR products cannot differentiate between narrow-spectrum and extended-spectrum enzymes of the TEM and SHV series. Other methods such as isoelectric focusing analysis (IEF) may just indicate the presence of acquired ß-lactamases rather than to identify an ESBL precisely. For example, PER-1, and narrow-spectrum TEM-1 enzymes share very similar pI values (39, http://www.lahey.org/studies). Primers designed for annealing to the ends of class 1 integrons may also help for obtaining PCR products that may contain ESBL encoding genes. Nucleotide sequence analysis of PCR products, whether or not combined with other methods (24), is still the only acceptable way to accurately discriminate between ESBL genes of the same family.

2.5 Clinical consequences.

The most appropriate antibiotic regimen for treating infections due to ESBL-positive *P. aeruginosa* strains remains to be determined due the lack of clinical studies in this research field. Three reports detail antibiotic therapy and outcome of patients infected with ESBL-positive *P. aeruginosa* isolates (17, 30, 51). Experimental pneumonia conducted in rats with a PER-1-producing *P. aeruginosa* strain (35), indicated that a combination of amikacin and imipenem was synergistic against an imipenem- and amikacin-susceptible strain. As predicted by results of in-vitro susceptibility testing, cefepime and piperacillin-tazobactam exhibited marked inoculum effects in vivo (35) and as previously documented for ESBL-producing *Enterobacteriaceae* (55), these results indicate that PER-1-producing *P. aeruginosa* would not be treated safely with piperacillin-tazobactam or cefepime alone (35). A population-based cohort study conducted with PER-1-producing *P. aeruginosa* in Turkey (61), identified the following factors as independent predictors of poor clinical outcome namely: i)

impaired consciousness, ii) male sex, and iii) urinary tract infection. Other clinically significant variables in that study were the presence of a central venous catheter, the acquisition of the infection in ICU, and hypotension. Unfortunately, the authors did not comment on the antibiotic regimen used in the study (61). Clinical experience with VEB-1-positive *P. aeruginosa* strains may indicate the efficacy of imipenem-containing antibiotic regimens as target therapy (17).

In a documented outbreak of a GES-2-producing *P. aeruginosa* strain that had occurred in South Africa, seventy-two ceftazidime-resistant isolates originating from nine patients were collected carrying $bla_{\text{GES-2}}$ and $bla_{\text{OXA-5}}$ genes (51). Out of these nine patients, only one had been hospitalised in a general gynaecology ward. The patient had a short stay in hospital after successful treatment of a wound infection with topical application of an acetic acid solution. The other patients had chronic underlying conditions, associated with extended periods in ICU and multiple courses of broad-spectrum antibiotics. Eight clonally related isolates tested resistant to most common beta-lactams (except aztreonam), aminoglycosides and ciprofloxacin. A total mortality of 62.5% (5/8 patients) was recorded, and the most successful treatment regimens consisted of combinations of imipenem with ciprofloxacin or aztreonam with amikacin, respectively (51). Piperacillin-tazobactam proved to be ineffective, even in combination with amikacin, against GES-2-producing *P. aeruginosa* (51). Retrospectively, this could be due to the relative resistance of this enzyme to beta-lactamase inhibitors (Table 2-3). Proof of invasive infections during this outbreak could be established by culturing GES-2-producing isolates from: i) blood cultures, ii) tissue biopsies and iii) arterial catheter tips (51). The outbreak was effectively terminated by: i) increasing the hygiene and housekeeping measures in ICUs, ii) restricting movement of patients infected or colonised with multiple-resistant *P. aeruginosa* isolates, and iii) increasing

the turn-over of patients hospitalised in these ICUs (G. F. Weldhagen, personal data). Use of topical non-absorbable antibiotics given orally such as colistin (59), for controlling enteric reservoirs of ESBL-positive enterobacterial isolates, has not been evaluated in the case of ESBL-positive *P. aeruginosa* isolates.

MIC results may help in choosing the optimal antibiotic regimen, but in-vitro susceptibility does not always guarantee in-vivo success. If the ESBL-positive isolate remains susceptible to carbapenems, a carbapenem should be proposed in combination with an antibiotic molecule of another non-beta-lactam class. Meropenem unlike imipenem remains stable to the hydrolytic activities of all class A ESBLs found in *P.aeruginosa*, including beta-lactamase GES-2 (52). Colonized skin wounds should not be treated with systemic antibiotics but rather dressed with topical applications of antiseptics. Increased general hospital hygiene measures as reported for controlling outbreaks of ESBL-positive enterobacterial isolates is a crucial point in controlling outbreaks due to ESBL-positive *P. aeruginosa.*

2.6 Conclusion.

Some ESBL-producing *P. aeruginosa* strains seem to be widely scattered in certain geographic locations such as VEB and PER enzymes in Southeast Asia and Turkey, respectively. Detection of other ESBLs in other countries may however reflect selective laboratory research interests rather than true distribution of these enzymes in *P. aeruginosa*.

High levels of frequency of detection of ESBLs in *P. aeruginosa* are from countries that have not yet implemented molecular-based surveillance programs for antibiotic resistance detection. Difficulties in laboratory detection of ESBLs and thus underreporting may likely increase the incidence and the prevalence of these enzymes

worldwide, especially in developing countries. In several cases, the current high prevalence of ESBLs in *P. aeruginosa* in those countries may be the source of transfer of ESBL-producing *P. aeruginosa* to developed Western countries as well as a hidden reservoir for ESBL genes transferred to other gram-negative aerobes. Since *P. aeruginosa* is known to be a formidable pathogen for acquiring additional resistance mechanisms, one should be aware that a multi-drug resistance trend would be very difficult to reverse in this species. The report of beta-lactamase GES-2 that is a weak carbapenem-hydrolyzing beta-lactamase raises an additional threat of selection of beta-lactamases with a very broad substrate profile and increased inhibitor resistance from ESBLs.

Reports of structurally related, integron-located ESBL genes in *P. aeruginosa* from different parts of the world, add novel steps in the saga of evolutive transfer of beta-lactamase-mediated antibiotic resistance, with questions arising on the origin of these genes. Additionally, co-resistance and co-expression of resistance determinants as the result of their integron location may further stabilize non-related antibiotic resistance genes. In other words, antibiotic regimens that may contain rifampin (60) or aminoglycosides for example may enhance prevalence of genes encoding resistance to structurally unrelated antibiotic molecules including expanded-spectrum cephalosporins and carbapenems (beta-lactamase GES-2). Thus, changes in antibiotic policy may include not only extended-spectrum cephalosporins, but also non-beta-lactam antibiotics and should involve discussions that include representatives of various clinical disciplines. Since the natural reservoir of many ESBL genes found in *P. aeruginosa* is unknown as well as the origin of the integron-located beta-lactamase genes, further research should be directed to search for these natural ESBL producers.

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