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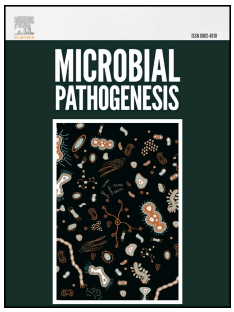

P. aeruginosa blood stream infection isolates: A “full house” of virulence genes in isolates associated with rapid patient death and patient survival

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P. aeruginosa blood stream infection isolates: A "full house" of virulence genes in isolates associated with rapid patient death and patient survival.

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Abstract

We have recently characterised the epidemiology of *P. aeruginosa* blood stream infection (BSI) in a large retrospective multicentre cohort study [1]. Utilising corresponding patient BSI isolates we aimed to characterise the genotypic virulence profile of the *P. aeruginosa* isolates that were associated with rapid death in the non-neutropenic host. Five *P. aeruginosa* BSI episodes were identified from a larger cohort of *P. aeruginosa* BSI episodes previously described by McCarthy *et al* [1]. . The genotypic profile of another 5 isolates from this cohort in whom the non-neutropenic host had survived one year post the BSI was also analysed for comparison. These isolates underwent Illumina whole genome sequencing and were de novo assembled using Spades VX. Annotation was performed by the NCBI prokaryotic Genome Annotation Pipeline. A comprehensive suite of virulence genes was determined by utilising the Pseudomonas Genome Database (http://www.pseudomonas.com/) [2]. . For a gene to be considered present searching required 100% of the gene to be present in the BLAST output. For both the Exolysin (ExlA) protein and the ExoU protein the reads were mapped in CLC Genomics Workbench V7.5.1 to the exlA (allele PSPA7_RS22085 in PA7 genome) or exoU (PA14_51530 allele in the PA14 genome). There was extensive conservation of virulence genes across all of the BSI isolates studied. The *exoU* gene was found in two patients who died rapidly and in one patient that survived one year post BSI. The *higA* and *higB* genes were detected in all isolates. The *exlA* gene was not detected in any of the isolates studied. These findings suggest that to cause a BSI that it is only the highly virulent *P. aeruginosa* isolate that succeeds. However the genetic armentarium is only one of many factors that result in death of the host. Further phenotypic correlation is required.

1.1 Highlights

- There was extensive conservation of virulence factors in all the *P. aeruginosa* BSI isolates studied
- Genes making up the TTSS and the *higA* and *higB* genes were identified in all BSI isolates
- The *exlA* gene was not identified in any of the BSI isolates studied.

1.2 Key Words: Pseudomonas aeruginosa; blood stream infection; bacteremia; virulence; mortality

1.3 Abbreviations

BSI Blood stream Infection

ExlA Exolysin protein

TTSS Type 3 protein secretion system

1.4 Introduction

With the utilisation of whole genome sequencing we have been able to gain greater understanding of the genome of *P. aeruginosa.* It is a large genome of 6.3 MB, containing on average more than 6175 genes [3]. The core genome represents on average 88% of the genome [4]. In comparison to the conserved core genome, the regions of the accessory genome are heterogeneous and contain genes identified from other bacteria [5]. Valot *et al* found that approximately one third of virulence genes are found in the accessory genome, the rest sitting in the core genome [4].

The virulence genes of *P. aeruginosa*, outside of the cystic fibrosis setting, have been studied in a limited fashion in such cohorts as burn infections, urinary tract infections and blood stream infections (BSI) [6-8]. More recently Pena *et al* studied a large cohort of patients with *P. aeruginosa* BSI [9]. They were able to show that the genotypic presence of *exoU* was significantly associated with early death. This was found to be more significant than the traditional patient risk factors also studied [9]. Elsen *et al* have recently described a toxin that causes hyper virulence in *P. aeruginosa* called ExlA [10]. This induces plasma membrane rupture in host cells [11]. Study in the context of patient outcomes has not been studied thus far.

We have recently characterised the epidemiology of *P. aeruginosa* BSI in a large retrospective multicentre cohort study [1]. Utilising corresponding patient BSI isolates we wished to characterise the genotypic virulence profile of the *P. aeruginosa* isolates that caused rapid death in the non-neutropenic host.

1.5 Methods

1.5.1 Study population

P. aeruginosa BSI's were studied retrospectively in a multicentre cohort study from the first of January 2008 to the first of January 2011 [1]. A BSI episode was identified as the 14 day time period from the date of the first positive blood culture. Five hundred and ninety-five episodes of *P. aeruginosa* BSI were identified over the three year period. After predefined exclusion criteria were applied, 388 monomicrobial non-recurrent BSI episodes from 388 patients were available for further analysis. This has been described in detail elsewhere [1].

At 48 hours post the collection of the sentinel blood culture 17 patients, representing 17 BSI episodes had died [1]. Of these patients, six were neutropenic within the 14 day period prior to the BSI episode and were excluded from this virulence study. In one patient their neutrophil count was not known and they were also excluded. Of the remaining ten BSI episodes, five of the BSI isolates had been stored by the servicing laboratory and were available for further study.

To obtain BSI isolates for comparison from non-neutropenic patients who had survived one year post BSI, 180 BSI episodes were identified from the previously mentioned cohort. Five BSI episodes were selected randomly from the cohort. The relevant BSI isolates had been stored by the servicing laboratory and were available for further study.

1.5.2 Microbiological Methods

Blood cultures were undertaken by the routine diagnostic laboratories using the BD BACTEC™ (43003-1) blood culture system (BD, North Ryde, Australia) with an incubation period of up to five days. Upon detection each culture positive blood sample was inoculated onto blood, chocolate and MacConkey agars and then incubated at 35^0C in either 5% CO_2 or aerobic conditions. After overnight incubation the *P. aeruginosa* isolates were identified by the VITEK 2 system (bioMérieux Australia Pty Ltd, Baulkham Hills, Australia). Confirmed

P. aeruginosa isolates were stored at $^{-}80^{0}$C using the Protect Microorganism Preservation System (Thermo Fisher Scientific Australia Pty Ltd, Thebarton, Australia) until transportation to a research laboratory. Upon receipt each isolate was resuscitated from $^{-}80^{0}$C storage on Mueller-Hinton agar at 37^{0}C for 24 hours. Antimicrobial susceptibility testing was performed by a microdilution method on the VITEK 2 system. Clinical and Laboratory Standards Institute breakpoints were used to define susceptibility or resistance to aztreonam, ticarcillin-clavulanate, piperacillin-tazobactam, ceftazidime, cefepime, meropenem, ciprofloxacin and gentamicin [12].

1.5.3 Whole Genome Sequencing and Analysis

Paired end libraries of whole genomic DNA (QIA symphony DSP DNA Mini kit) of all 5 isolates were prepared by the Nextera library protocol and sequenced by Illumina HiSeq2000 or NextSeq500 (Illumina, San Diego, CA, USA). Sequences were trimmed in CLC Genomics Workbench 7.5.1. All sequences were *de novo* assembled using SPAdes 3.8.1. Annotation was performed by the NCBI prokaryotic Genome Annotation Pipeline. The draft genomes were 4,654,913 to 6,618,287 bp long consisting of 112 to 400 contigs. (MWZF00000000.1 Total number of genes: 6520 , CDS 6248, tRNA 50, non-coding RNA 4; MWZG00000000.2 Total number of genes: 5197, CDS 5143, tRNA 47 and non-coding RNA 3; MWZH00000000.1 Total number of genes: 5380 , CDS 5168, tRNA 43, non-coding RNA 4; MWZI00000000.1 Total number of genes: 4563 , CDS 4512, tRNA 44 and non-coding RNA 4; MWWL00000000.1 Total number of genes: 6502 , CDS 6440, tRNA 55, non-coding RNA 4; SUB 3659983, SUB3660005, SUB3660009, SUB3660020, SUB3660023. Contigs of the draft genomes were submitted to the Centre for Genomic epidemiology (http: //www.genomic epidemiology.org) to identify the resistance genes of each isolate with the database Resfinder 2.1 [13] . The sequence type (ST) of each isolate was also identified by the *Pseudomonas*

aeruginosa MLST database 1.7 [14]. Two novel ST sequences were named upon submission to the MLST website (https://pubmlst.org/wolbachia/)[15]. A comprehensive suite of instrinsic virulence genes, found in wildtype PA01, was determined by utilising the Pseudomonas Genome Database (http://www.pseudomonas.com/) [2]. For the HigB toxins, primer sequences were obtained and blasted to identify the gene product [16]. For a gene to be considered present searching required 100% of the gene to be present in the BLAST output. For both the Exolysin (ExlA) protein and the ExoU protein the reads were mapped in CLC Genomics Workbench V7.5.1 to the exlA (allele PSPA7_RS22085 in PA7 genome) or exoU (PA14_51530 allele in the PA14 genome).

1.6 Results

The first five *P. aeruginosa* isolates studied caused BSI in a non-neutropenic patient who experienced death within 48 hours of collection of the blood culture from which the bacteria was identified. The host studied was not free of co-morbidities with two of the patients having metastatic small cell lung cancer and one patient having chronic lymphocytic leukaemia. Two of the patients had received chemotherapy in the preceding 30 days and three patients recent corticosteroid therapy. The BSI episodes were monomicrobial, non-recurrent and phenotypically susceptible to all the antibiotics tested. All BSI isolates originated from a presumed lung source of infection. In two BSI episodes the patients did not receive empirical therapy that would have adequately covered the infecting *P. aeruginosa* isolate (Table 1, isolates 1-5).

In comparison, the further five BSI isolates studied caused BSI in a non-neutropenic patient who was alive at one year post the collection of the blood culture from which the bacteria was identified. Multiple host co-morbidities were again seen. Three of the five patients had either

an oncological or haematological condition. Only one patient had received chemotherapy in the preceding 30 days and one patient corticosteroids. All the BSI episodes were monomicrobial, non-recurrent and phenotypically susceptible to all antibiotic tested. Three isolates originated from a lung source of infection and two isolates from a urinary tract infection. All patients received empirical therapy that would have adequately covered the infecting *P. aeruginosa* isolate (Table 2, isolates 6-10).

Table 1: Patient characteristics of the five P. aeruginosa BSI episodes associated with rapid patient death

	Patient Number	Age	Sex	Acquisition	Source	Pitt Score	Co-morbidities	Medical Therapy in the last 30 days	Empirical Therapy
Death within 48	1	73	F	HCAI	Pneumonia	1	Metastatic NSCLC (Lung primary)	Chemotherapy, corticosteroids	Gentamicin, cefotaxime
	2	54	M	HCAI	Lung Source	2	Metastatic NSCLC (Lung primary)	Corticosteroids	Gentamicin, timentin, azithromycin
	3	86	M	HCAI	Lung source	12	Dementia, Chronic renal failure, Interstitial Lung Disease, atrial fibrillation	Corticosteroids	Ceftriaxone, azithromycin
	4	88	M	HCAI	Pneumonia	6	Asthma, COPD, hypertension, atrial fibrillation		Benzylpenicillin, ceftriaxone, azithromycin
	5	89	F	HCAI	Pneumonia	2	Lung Cancer, Chronic lymphoctic leukaemia, hypertension	Chlorambucil	Vancomycin, gentamicin
Alive 1 year	6	64	M	HAI	Urinary Tract Infection	3	Prostate Cancer, Gout, COPD, IHD		Ceftazidime
	7	63	F	CAI	Pneumonia	5	Myeloma, Hypertension, GORD		Timentin, gentamicin
	8	93	F	HAI	Pneumonia	2	Asthma, IHD, Hypertension		Timentin
	9	66	M	HCAI	Pneumonia	3	Myelofibrosis, Sweets Syndrome, COPD, Hypertension	Corticosteroids	Timentin, gentamicin
	10	49	M	HAI	Urinary Tract Infection	1	Recent brain trauma and secondary bleed		Timentin, ciprofloxacin

Footnote 1: NSCLC (Non small cell lung cancer), COPD (Chronic obstructive pulmonary disease

The ST types identified across the tenisolates were all unique and in two instances comprised novel genotypes on the PubMLST database. The genotypic antibiotic resistance profiles of each isolate are also described in Table 2. Phenotypically the isolates were all found to be susceptible to ceftazidime, cefepime, ticarcillin-clavulanate, piperacillin-tazobactam, meropenem, gentamicin and ciprofloxacin.

Table 2: MLST and antibiotic resistance genotype of the ten BSI isolates studied

	Death within 48 hours					Alive 1 year				
Isolate Number	1	2	3	4	5	6	7	8	9	10
MLST type	ST-569	ST-309	ST-898	Unknown ST	Unknown ST	ST-1639	ST-244	ST-671	ST-12	ST-931
Antibiotic Resistance										
Genotypic										
Aminoglycoside	aph(3')-2b	aph(3')-2b	aph(3')-2b	aph(3')-2b	aph(3')-2b	aph(3')-2b	aph(3')-2b	aph(3')-2b	aph(3')-2b	aph(3')-2b
Betalactamase	blaOXA-50	blaOXA-50	blaOXA-50	blaOXA-50	blaOXA-50	blaOXA-50	blaOXA-50	blaOXA-50	blaOXA-50	blaOXA-5(
	blaPAO	blaPAO	blaPAO	blaPAO	blaPAO	blaPAO	blaPAO	blaPAO	blaPAO	blaPAO
Chloramphenicol	catB7	catB7	catB7	catB7	catB7					
Fosfomycin	fosA	fosA	fosA	fosA	fosA	fosA	fosA	fosA	fosA	fosA
Phenotypic resistar	Fully susceptible	Fully susceptible	Fully susceptible	Fully susceptible	Fully susceptible	Fully Susceptible	Fully Susceptible	Fully Susceptible	Fully Susceptible	Fully Susc

Footnote 2: Multilocus sequence type (ST)

The genotypic virulence profiles of the isolates were determined as per Table 3. There was extensive conservation of virulence genes across all ten of the BSI isolates studied. Threeof the isolates contained the *exoU* gene. The *higA* and *higB* genes were detected in all isolates. The *exlA* gene was not detected in any of the isolates studied.

Table 3: Genotypic virulence profile of the five BSI isolates studied

			Isolates associated with rapid patient death					Isolates associated with patient survival				
System	Virulence Factor	Gene	1	2	3	4	5	6	7	8	9	10
Type 1 Secretion System	Alkaline phosphatase	*aprF*	+	+	+	+	+	+	+	+	+	+
	Heme acquistion protein	*hasAP*	+	+	+	-	+	+	+	+	+	+
Type 2 Secretion System	Elastase	*lasB*	+	+	+	+	+	+	+	+	+	+
	Protease Precursor	*lasA*	+	+	+	+	+	+	+	+	+	+
	Non-hemolytic phospholipase C precursor	*pcLN*	+	+	+	+	+	+	+	+	+	+
	Protease	*piv*	+	+	+	+	+	+	+	+	+	+
	Lactonising lipase precursor	*lipA*	+	+	+	+	+	+	+	+	+	+
	Lipase	*lipC*	+	+	+	+	+	+	+	+	+	+
	Exotoxin A precursor	*toxA*	+	+	+	-	+	+	+	+	+	+
	Low-molecular weight alkaline phosphatase	*lapA*	+	-	+	+	-	+	+	+	+	+
	Metalloendopeptidase	*mep72*	+	+	+	+	+	+	+	+	+	+
Type 3 Secretion System	Exoenzyme S	*exoS*	+	-	+	+	-	+	+	-	+	+
	Adenylate cyclase ExoY	*exoY*	+	-	+	-	-	+	+	+	+	+
	Exoenzyme T	*exoT*	+	+	+	+	+	+	+	+	+	+
	Exoenzyme U	*exoU*	-	+	-	-	+	-	-	+	-	-
	Secreted regulator of type III secretion gene	*exse*	+	+	+	+	+	+	+	+	+	+
Type 4 Secretion System	Amidase	*tse1*	+	+	+	+	+	+	+	+	+	+
	Type 4 secretion system effector	*tse2*	+	+	+	+	+	+	+	+	+	+
	Muramidase	*tse3*	+	+	+	+	+	+	+	+	+	+
	Pyoverdin	*vgrG*	+	+	+	+	+	+	+	+	+	+
	3-oxo-C12-homoserine lactone acylase	*pvdQ*	+	+	+	+	+	+	+	+	+	+
Type 5 Secretion System	Esterase	*estA*	+	+	+	+	+	+	+	+	+	+
	Adhesive protein	*cupB5*	+	+	+	+	+	+	+	+	+	+
	Exoprotease	*lep*	+	+	+	+	+	+	+	+	+	+
Toxin/Antitoxin System	Toxin/antitoxin system	*higA*	+	+	+	+	+	+	+	+	+	+
	Toxin/antitoxin system	*higB*	+	+	+	+	+	+	+	+	+	+
Biofilm	Alginate biosynthesis protein	*algX*	+	+	+	+	+	+	+	+	+	+
	GDP-mannose 6-hydrogenase	*algD*	+	+	+	+	+	+	+	+	+	+
	Poly(beta-d-mannuronate) lyase precursor	*algL*	+	+	+	+	+	+	+	+	+	+
	Alkaline metalloproteinase precursor	*aprA*	+	+	+	+	+	+	+	+	+	+
Lectins	Fructose-binding lectin PA-IIL	*lecB*	+	+	+	+	+	+	+	+	+	+
Neuramindases	Pseudoaminidase	*nan1*	+	-	+	+	+	+	+	+	+	+
	Pseudoaminidase	*nan2*	+	+	+	+	+	+	+	+	+	+
Quorum Sensing Proteins	Autoinducor synthesis protein	*lasL*	+	+	+	+	+	+	+	+	+	+
	Transcriptional regulator	*lasR*	+	+	+	+	+	+	+	+	+	+
Rhamnolipids	Autoinducor synthesis protein	*rhll*	+	+	+	+	+	+	+	+	+	+
	Transcriptional regulator	*rhlR*	+	+	+	+	+	+	+	+	+	+
Flagella	Flagellin type B	*fliC*	+	-	+	+	+	+	+	+	+	+
Polysaccharides	Polysacharides	*psL*	+	+	+	+	+	+	+	+	+	+
Excreted factor	Hydrogen cyanide	*HCN*	+	+	+	+	+	+	+	+	+	+
ExlA Secretion System	Exolysin	*exlA*	-	-	-	-	-	-	-	-	-	-

1.7 Discussion

The pathogenesis of *P. aeruginosa* is multifactorial as suggested by the large number of virulence determinants of this bacterium. We have characterised the virulence gene profile of five BSI isolates associated with rapid patient death by whole genome sequencing. This has been compared to the virulence gene profile of the BSI isolates of five patients that were alive at one year post BSI. Almost complete conservation of an extensive set of virulence genes was seen in both the isolates associated with patient death and also survival. It may be that to cause an invasive infection it is only the fittest isolate that succeeds. Futher phenotypic correlation is required to determine if there is any difference in genotypic expression by the BSI isolates in the two cohorts described.

The characteristics of the larger patient cohort from which the BSI episodes came from is described in detail [1]. This was a patient cohort with significant co-morbidities, predominantly cardiovascular or haematological in origin and in whom receipt of medical therapy in the preceding 30 days occurred in 221 (57%) of the patients. The most common therapy being either chemotherapy or corticosteroids [1]. Thus the patient characteristics of the five BSI episodes in this study associated with rapid death of the host have also been described in patients who did not die in the year post BSI. We know from the literature that a higher PITT score, as seen in patient three, predicts a high risk of death from the BSI [17]. Also that a lung source would be considered a high risk bacteraemia for mortality [18]. However to put this in context of the larger cohort described, not all patients with a lung source of BSI died [1]. The empirical therapy was considered inadequate in four of the five BSI's associated with rapid host death that were studied [1]. The importance of adequate empirical therapy is still debated in the setting of this infection in the literature [19-21]. Antibody protection to *P. aeruginosa* infection has also been described in animal models [22, 23]. As such although the patients described that experienced rapid death would be higher

risk for death in the setting of this BSI, the BSI isolate must also play a role. What are the genotypic virulence characteristics of the *P. aeruginosa* BSI isolate that is associated with rapid death?

The literature has focused on the type 3 protein secretion system (TTSS) and virulence in *P. aeruginosa* infections. El-Sohl *et al* performed a retrospective analysis of 85 cases of *P. aeruginosa* BSI at a tertiary care hospital. Bacterial isolates were assayed *in vitro* for secretion of type 3 exotoxins. They found that at least one of the TTSS proteins was detected in 37 of the 85 isolates (44%). In addition the overall 30 day mortality was significantly greater on multivariate analysis in those isolates that were TTSS positive [24]. Pena *et al* looked at the genotypic relationship of the TTSS system in *P. aeruginosa* BSI's and found a significant relationship between early mortality for the *exoU* genotype. This was also associated with a moderately resistant susceptibility profile [9]. Only two of the five BSI isolates that were isolated from a patient with early mortality were *exoU* positive in the current study. One of the five *P.aeruginosa* BSI isolates associated with patient survival at one year was also found to contain this gene.

Recently two other significant toxins as a cause of virulence in this pathogen have been described. A novel toxin *exlA* was also identified by Elsen *et al*, which induces plasma rupture in host cells [10]. Reboud further studied 18 isolates, including one BSI isolate, that contained this toxin and was TTSS negative [11]. They found that the behaviour and virulence traits of the *exla+* strains were variable in the mouse and chicory leaf model [11]. This toxin gene was not found in any of the BSI isolates we studied. Wood et al have also recently characterised the HigB/HigA toxin-antitoxin system. The HigB toxin was shown to reduce pyocynanin, pyochelin, swarming and biofilm formation [16]. The *higA* and *higB* genes were found in all the BSI isolates studied.

Focusing on acute infections caused by *P. aeruginosa*, limited numbers of genes related to pathogenicity have been described from different types of clinical isolates. Consistent gene conservation has not been seen. Faraji *et al* looked at burn wound isolates by targeted PCR and found that the prevalence rate of the virulence factors was as follows: *tox A* (36.9%), *algD* (70.1%), *plcH* (79%), *plcN* (63.1%), *lasB* (82%) and *exoS* (21.1%) [6]. Sebharwal *et al* looked at isolates from catheter associated urinary tract infections by targeted PCR and found that the prevalence rate of virulence factors was *toxA* (100%), *plcH* (75%), *lasB* (75%), *lasI* (75%), *lasR* (75%), *rhlI* (41.6%), *rhlR* (58.3%), *aprA* (16.6%), *rhlAB* (50%), *fliC* (58.3%) [7]. We found a high degree of conservation of the virulence genes in all the BSI isolates studied. This was not dependent on whether the host died rapidly or not. It may be that to cause a blood stream infection that it is only the fittest isolate that succeeds. Wolfgang *et al* looked at 18 *P. aeruginosa* strains from both the environment and clinical samples. Two isolates of which were from blood. Utilising whole-genome DNA microarray they found that upon examination of a gene set predicted to play a role in virulence, there was a high level of conservation across the isolates (97%) [25]. Our study looking at BSI isolates supports this finding.

The traditional view of *P. aeruginosa* pathogenicity was that virulence factors were the most important factors in the infective process. Recent work by Turner *et al* involving translation research in mouse models has changed the thinking relating to virulence [26]. This study combined both mutant fitness profiling and transcriptome profiling to study which *P. aeruginosa* genes were important for acute or chronic infections. For most classes of genes, including virulence genes, this analysis found that there was little association between transcript abundance and the contribution of the corresponding gene towards overall fitness. However for certain metabolic genes these factors were strongly associated. Interestingly it would seem that it is the environment that *P. aeruginosa* finds itself in that drives the

metabolic pathways and subsequent expression of virulence factors as part of a process of survival.

Limitations of the current study include lack of correlation of phenotypic expression of virulence with the genotypic findings. It would have also been ideal if a larger selection of BSI isolates could have been studied.

1.8 Conclusions

In conclusion this study places the genotypic detection of virulence factors of *P. aeruginosa* BSI isolates in the clinical context. Five *P. aeruginosa* BSI isolates that were associated with rapid patient mortality have had characterisation of their virulence gene profile. Five *P. aeruginosa* BSI isolates that were associated with patient survival at one year were also characterised as a comparator group. There was extensive conservation of virulence genes across the isolates. Threeof the isolates contained the *exoU* gene which was not limited to the host who experienced rapid death. The *higA* and *higB* genes were detected in all isolates. The *exlA* gene was not detected in any of the isolates studied. These findings may reflect that to cause both a blood stream infection that it is only the highly virulent *P. aeruginosa* isolate that succeeds. Futher phenotypic correlation is required to determine if there is any difference in genotypic expression by the BSI isolates that were associated with rapid death of the host and those BSI isolates associated with host survival at one year.

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1.10 Conflicts of interest: none

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1.1 Highlights

- There was extensive conservation of virulence factors in all the *P. aeruginosa* BSI isolates studied
- Genes making up the TTSS and the *higA* and *higB* genes were identified in all BSI isolates
- The *exlA* gene was not identified in any of the BSI isolates studied.