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ORIGINAL ARTICLE

# Virulence factors and infection ability of *Pseudomonas aeruginosa* isolates from a hydropathic facility and respiratory infections

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## Abstract

**Aims:** To compare the virulence pool and acute infection ability of *Pseudomonas aeruginosa* isolates from a hydropathic facility, used to treat respiratory conditions by inhalation of untreated natural mineral water, with clinical isolates from respiratory infections.

**Methods and Results:** *Pseudomonas aeruginosa* isolates from a hydropathic facility and from respiratory infections were typed by pulsed-field gel electrophoresis. Nonclonal representatives of each population were selected. 18 virulence-encoding genes were screened by polymerase chain reaction and statistically compared by multiple correspondence analysis. Homogeneous distribution of genes between populations but higher genetic association in aquatic isolates was observed, as well as distinct virulence pool according to location in the water system. Acute infection ability of selected isolates from each population, in *Galleria mellonella* model, showed lower $LD_{50}$ of the majority of the hydropathic isolates and significant variations in $LD_{50}$ of biofilm isolates from different equipments.

**Conclusions:** Hydrotherapy *Ps. aeruginosa* isolates present similar virulence to isolates from respiratory infections. Hydrotherapy users may be exposed to different microbiological risks when using different treatment equipments.

**Significance and Impact of the Study:** Twenty-one million people use hydropathic facilities in Europe, and the majority present risk factors to pneumonia. This study demonstrates the health risk associated with this practice. Revision of European regulations should be considered.

## Introduction

Hydrotherapy is a natural therapeutic practice that uses natural mineral water (NMW) to treat several health conditions, including respiratory-associated illnesses such as asthma, bronchitis, emphysema and bronchiectasis, in facilities commonly named *thermae*. This natural practice is very frequent in Europe, with more than 21 million users (Pereira *et al.* 2011). Treatments are performed using equipments similar to the hospital ventilators, and *thermae* users deep inhale aerosol particles of undisinfected NMW for about 15–20 min on a daily basis, typically during 7–14 consecutive days. At present, there are no European directives specifically regulating the use of NMW in hydropathic establishments. The only information available from the European Council regarding the definition and quality control assessment of NMW is present in European Directive 2009/54/CE (European Parliament and Council 2009). In its annex I, the regular survey of *Pseudomonas aeruginosa* is mandatory, but only

in its planktonic form, neglecting the biofilm forms, which are the prevalent ecological forms present in environmental settings (Ma *et al.* 2009). A previous study demonstrated the presence of *Ps. aeruginosa* biofilm isolates in *thermae* respiratory treatment equipments, with an increased potential to acquire antimicrobial resistance (Pereira *et al.* 2011).

*Pseudomonas aeruginosa* is a ubiquitous environmental bacterium that can be found in water, soil and plants, infecting many different organisms such as yeasts, plants, nematodes, insects and mammals (Battle *et al.* 2009). In humans, *Ps. aeruginosa* is the most frequent infection agent isolated from the respiratory tract of immunocompromised patients, the leading cause of morbidity and mortality in patients with ventilator-acquired pneumonia and cystic fibrosis (Fricks-Lima *et al.* 2011), and responsible for a significant part of community-acquired pneumonia (Arancibia *et al.* 2002). The pathogenesis of *Ps. aeruginosa* pneumonia is complex and involves an arsenal of several secreted and cell-associated virulence factors. These include adhesins, proteases, phenazines and type III secretion system exotoxins (T3SS). Adhesins, such as lectins and motility features, participate in the initial stage of infection, allows the bacteria to adhere to the host cells and are cytotoxic to epithelial lung cells (Strateva and Mitov 2011). Proteases, mainly alkaline protease and elastase, degrade elastin, which represent 28% of the lung tissue (Okumura *et al.* 2008). Phenazines increase intracellular oxidative stress, inhibit mitochondrial activity and cell proliferation in neutrophiles and macrophages (Bradbury *et al.* 2010). T3SS promote eukaryotic cells apoptosis and dissemination of disease from the lung (Hauser 2009; Bradbury *et al.* 2010).

The majority of virulence factors are probably natural features of bacteria in order to avoid or survive predation made by many organisms that inhabit soil and water (Engel and Balachandran 2009; Hauser 2009). Some studies reported the occurrence of virulence factors in environmental *Ps. aeruginosa* isolates (Hauser 2009; Bradbury *et al.* 2010; Deptula and Gospodarek 2010), but the virulence pool of *thermae Ps. aeruginosa* isolates was not yet addressed. This assessment is important to better understand the risk that *thermae* health-compromised users are exposed to when using this natural therapy. The aim of this work was to study treatment equipment biofilms and planktonic *Ps. aeruginosa* isolates obtained from a hydropathic facility, regarding the presence and distribution of several virulence genes (VG) and its ability to cause acute infection. Comparison with clinical isolates associated with respiratory infections was made to better assess the virulence and infection potential of the *thermae* isolates.

## Material and methods

### Bacterial population

Seventy-seven *Ps. aeruginosa* isolates were randomly selected from a population of 226 isolates from a Portuguese *thermae* facility (Pereira *et al.* 2011): 26 isolates were from biofilms isolated from the inner parts of three respiratory treatment equipments (two aerosols and one collective nebulizer), and 45 were planktonic isolates collected from two boreholes, one main deposit and nine water distribution circuits. Fifty *Ps. aeruginosa* clinical isolates were included in the study for comparative purposes, randomly selected from sputum and lung aspirate samples of a *Ps. aeruginosa* culture collection from a Portuguese central hospital obtained from nosocomial and ambulatory infections.

### Pulsed-field gel electrophoresis (PFGE) fingerprinting

PFGE typing was performed according to Maslow *et al.* (1993) with minor modifications. All strains were digested with *SpeI* (Takara, Japan), and the resulting fragments were separated by electrophoresis in 1·2% agarose gels, using CHEF-DR III® apparatus (BioRad, Hercules, CA), in 0·5× Tris-borate-EDTA (TBE) running buffer, at 14°C and 6 V $cm^{-1}$, for 18 h with pulse time ranging from 1 to 25 s. Midrange PFG Marker II (New England BioLabs, Ipswich, MA) DNA pattern was used to allow calibration and normalization of gels. Gels were stained with SYBR Safe (Invitrogen, Carlsbad, CA), photographed under UV light and scanned. Resulting images were analysed using Quantity One® ver. 4.6.6 (BioRad), by automated detection and comparison of bands in each lane, allowing matching profiles to be generated, which were used to produce a dendrogram by applying the unweighted pair group method with arithmetic mean (UPGMA). Cut-offs to determine clonal relatedness was set at the level of similarity observed for the DNA patterns.

### Detection of virulence-encoding genes

Screening for the presence of genes *flaG*, *orfF*, *pilA*, *pilB*, associated with motility, *lecA*, *lecB*, involved in lectin production, *apr, lasA*, *lasB*, encoding protease and elastases, *phzH*, *phzM*, *phzS*, *phzI*, *phzII*, involved in the biosynthetic pathway of phenazines, and *exoS*, *exoT*, *exoU* and *exoY*, encoding T3SS, was carried out by polymerase chain reaction (PCR) using specific primers, as previously described (Allewelt *et al.* 2000; Finnan *et al.* 2004; Chemani *et al.* 2009; Kaszab *et al.* 2010). PCR was performed, individually for each pair of primers used to detect the genes above indicated, in a 34 subsequent

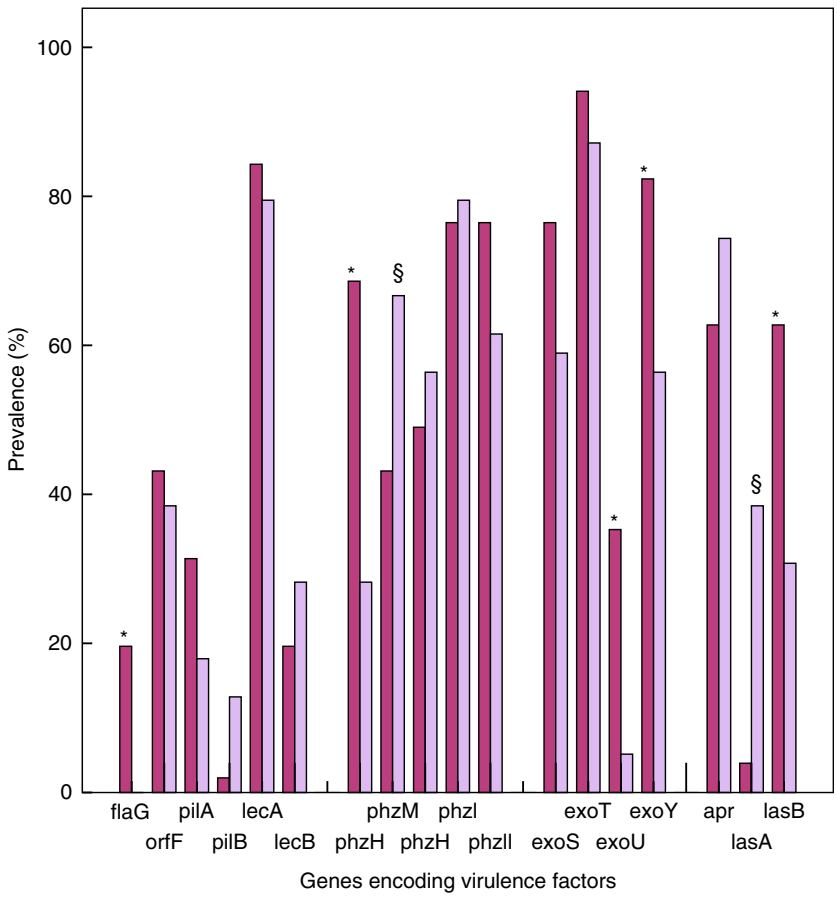


**Figure 1** Prevalence (%) of genes encoding virulence factors in *thermae* ($n$ = 51) and clinical ($n$ = 39) samples. Chi-square tests revealed a homogeneous distribution of genes *orfF*, *pilA*, *pilB*, *lecA*, *lecB*, *phzS*, *phzI*, *phzII*, *apr*, *exoS* and *exoT* ($P > 0{\cdot}05$) between populations; and a heterogeneous distribution, with prevalence in *thermae* population (*) of genes *flaG* ($P = 0{\cdot}002$), *phzH* ($P < 0{\cdot}001$), *exoU* ($P = 0{\cdot}007$), *exoY* ($P < 0{\cdot}001$) and *lasB* ($P = 0.002$). Genes *phzM* ($P = 0{\cdot}022$) and *lasA* ($P < 0{\cdot}001$) were more prevalent in the clinical isolates (§). (■) Thermae and (□) Clinical.

**Table 1** $LD_{50}$s of *thermae* and clinical *Pseudomonas aeruginosa* isolates, tested in the greater wax *Galleria mellonella* larvae model

| *Thermae* | | | | Clinical | | | |
|---|---|---|---|---|---|---|---|
| Code | Origin | $LD_{50}$ (CFU ml$^{-1}$) | Error | Code | Origin | $LD_{50}$ (CFU ml$^{-1}$) | Error |
| B12 | Equipment 1 | 4172 | 131 | 003/09 | Aspirate | 734 | 15 |
| B45 | Equipment 2 | 2034 | 35 | 012/09 | Sputum | 3607 | 24 |
| B41 | Equipment 3 | 458 | 57 | 095/09 | Aspirate | 2779 | 4 |
| F1 | Borehole 2 | 1243 | 18 | 005/09 | Exudate | 76401 | 1007 |
| C9 | Main deposit | 1267 | 22 | 042/09 | Urine | n.d.($LD_{100} < 8 \times 10^8$) | – |

CFU ml$^{-1}$, colony-forming unit per millilitre; n.d., not determined.
$LD_{50}$s estimates were determined by means of a four parameter logistic regression model based on the decimal logarithms of the mean results obtained in three independent experiments. Overall fit was assessed through $R^2_{adj}$ ($R^2_{adj} > 0{\cdot}99$) and $F$ test ($P$-value < 0·05).
Error was determined using the error propagation formula for base-10 power functions.

cycles of denaturation at 94°C for 30 s, annealing at 59·5, 64, 59, 54, 55·5, 59, 51, 47, 57, 51, 54, 63, 49, 51, 53, 58, 58 and 61·8°C, respectively, for 30 s and extension at 72°C for 30 s (the first denaturation cycle was at 94°C for 3 min and the final extension cycle at 72°C for 5 min), with expected fragments of 1689, 946, 1675, 478, 736, 836, 1017, 1075, 1220, 1752, 875, 1752, 392, 1036, 328, 152, 428 and 1035 bp, respectively. Sequencing of PCR products with expected size was performed, and results were analysed using BLAST®. A similarity ≥99·5% between the amplicons sequenced and previously reported sequences available in GenBank® was considered a positive result.

### *Galleria mellonella* killing assay

*Galleria mellonella* killing assays were performed as described in Miyata *et al.* (2003) with minor

modifications. Two biofilm isolates from two different equipments (collective nebulizer and aerosol 2) and two planktonic isolates (borehole 2 and main deposit), randomly selected from the subset of isolates from each location, and three clinical isolates, randomly selected from the studied sample, were essayed. One biofilm isolate from the third equipment not included in the virulence screen, and 2 clinical isolates from diverse origin (exudates and urine) were also included, for comparison. Strains were grown in LB medium overnight at 37°C. Bacterial suspensions were prepared with 10-fold serial dilution in 10 mmol $l^{-1}$ $Mg_2SO_4$ supplemented with ampicillin (1·2 mg $ml^{-1}$) and 3·5 $\mu$l injected into hindmost left proleg. Ten larvae were injected at each dilution, incubated at 37°C in the dark and scored death/alive after 48 h of infection. Control larvae were injected with 10 mmol $l^{-1}$ $Mg_2SO_4$ with ampicillin only. The results presented were obtained in 3 independent assays. The 50% lethal dose ($LD_{50}$) was determined for each strains under study using a nonlinear regression dynamic fitting from the mean results of three independent experiments, with iterations statistically validated by normality and constant variance tests ($P < 0·05$) using SYSTAT® SIGMAPLOT® program, ver. 11.0 (Chicago, IL). Associated error was estimated by error propagation formula of constant potency.

### Virulence data statistical analysis

Statistical analysis was performed using IBM® SPSS® Statistics, ver. 21.0 (Chicago, IL). Chi-square tests were used to infer on the homogeneity of VG distribution between *thermae* and clinical populations. Results were considered statistically significant at $P < 0·05$. Multiple correspondence analysis (MCA), a weighted principal component analysis method that allows the graphical understanding of simultaneous relations between several categories in a perceptual map (Greenacre 2008), was used to describe the overall dependence structure between VG modalities in the *thermae* isolates, with each other and with location, and to compare it to the one observed in the clinical sample. Chi-square tests of independency were used to infer on the statistical significance ($P < 0·05$) of the VG relations visualized in the MCA plots. Phi coefficient was used to measure the direction and strength of the associations, with $\Phi > 0·4$ considered a strong association and $0·2 < \Phi < 0·4$ moderate. Due to a < 10% modality observation (presence or absence), genes *pilB*, *lasA*, *exoT* and *exoU* were excluded from *thermae* MCA and *flaG*, *lecA* and *exoU* from clinical population MCA, according to the principles stated by Le Roux and Rouanet (2004).

## Results

### Population's genetic diversity evaluated by PFGE

Genetic relatedness of the 77 *thermae* and 50 clinical isolates selected for the study was surveyed by PFGE, and clones were excluded to eliminate bias in the data, retaining 51 isolates from *thermae* population (27 planktonic and 24 from two treatment equipments biofilms), and 39 clinical isolates (24 from sputum and 15 from aspirates).

### Virulence distribution and association

Both populations presented all the studied VG, except *flaG* in clinical population. *Thermae* population revealed, in general, higher VG rates than clinical isolates (Fig. 1), but the differences were not all statistically significant, with two-thirds of the studied genes homogeneously distributed between populations. Genes *flaG*, *phzH*, *exoY* and *exoU* were more prevalent in *thermae,* while genes *phzM* and *lasA* were the only VG with higher prevalence in the clinical isolates.

In both MCAs, we retained 3 dimensions, explaining 63·2 and 65% of total variance in *thermae* and clinical populations, respectively. Figures 2 and 3 present respective plots, with dimension 2 and 3 plot omitted due to lack of relevant information in it. All VG related with each other in a positive direction (presence relating to presence, or *vice-versa*), except *lecB* in *thermae* isolates (Fig. 2). Statistically significant associations ($P < 0·05$) of VG were highlighted in MCA plots with filled or dotted lines, according to their strength (Figs 2 and 3), to allow a better perception of the overall association of VG with each other, as chi-square tests only provide information of 2 × 2 associations (Table S1). The majority of the observed VG relations in *thermae* MCA were statistically significant, quantified as strong ($\Phi > 0·4$) or moderate ($0·2 < \Phi < 0·4$) (Table 1). In clinical isolates MCA, some VG whose modalities that were plotted together revealed to have no statistical significant association (Fig. 3); however, except for *lecA*, all detected associations were strong ($\Phi$ coefficient > 0·4), and 3 were very strong ($\Phi$ coefficient > 0·6) (Table S1).

Gene *lecA* in *thermae* isolates and genes *pilA*, *pilB*, *lecB*, *apr* and *lasB* in clinical isolates did not associate with any genes of respective samples. The genes involved in the biosynthesis of phenazines (*phzH*, *phzM*, *phzS*, *phzI* and *phzII*) were highly associated in both populations, with each other and with the majority of the other tested genes (Figs 2 and 3). Gene *phzI* was an exception in *thermae* population, plotted in opposite quadrants of other phenazine genes (Fig. 2), only associating with *exoS*, *exoY*, *apr*, *lasB* and *lecB* genes. T3SS-encoding genes

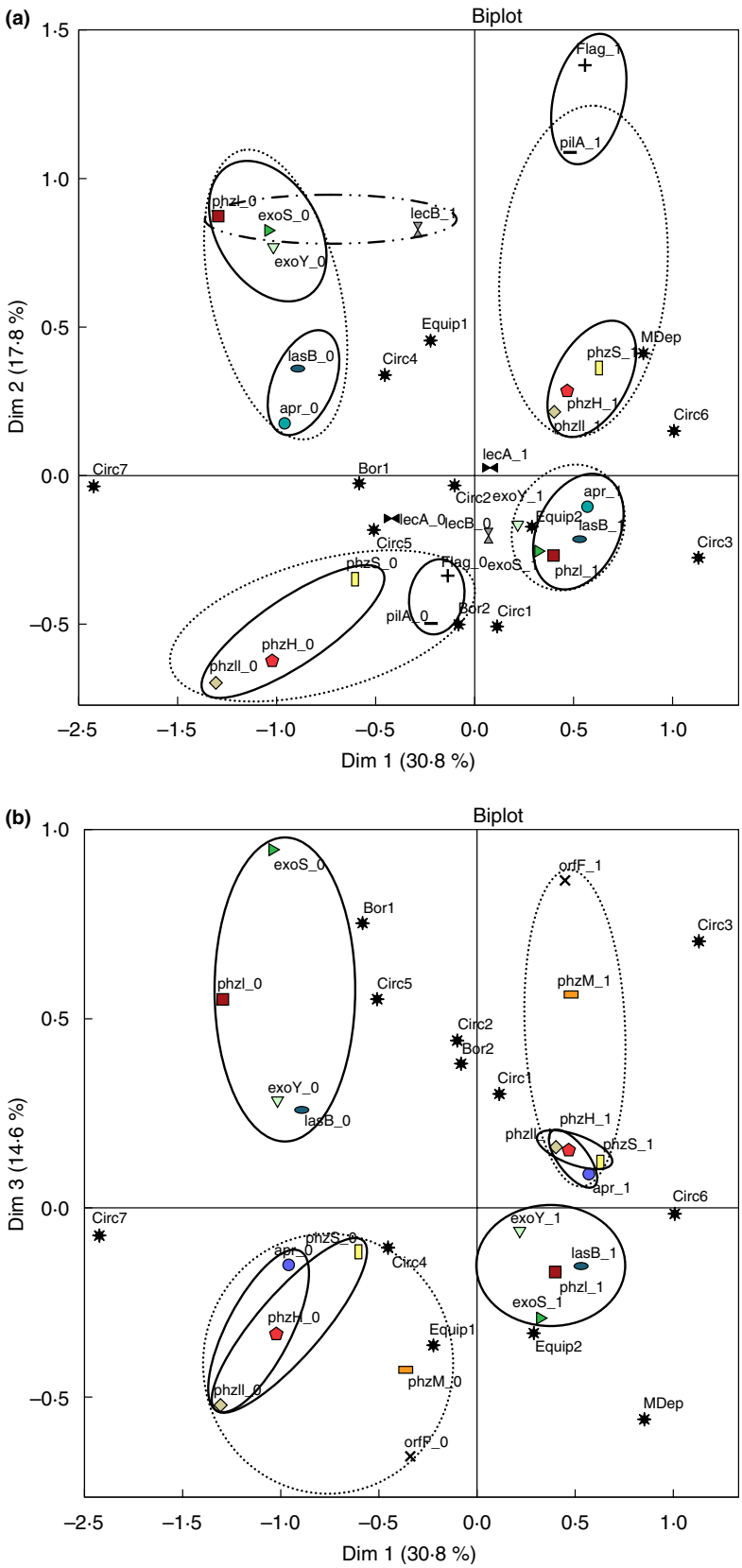


**Figure 2** MCA biplots of the *thermae* sample (a – dimension 1 + 2; b – dimension 1 + 3). In each quadrant, circles highlight relations of virulence-encoding genes that showed significant association ($P < 0{\cdot}05$). Circles with filled lines represent strong associations (Φ coefficient > 0·4). Circles with dotted lines represent weak associations (0·2 < Φ coefficient<0·4). In dimension 1 + 2 biplot, one circle represented by dots and lines indicate negative, weak, associations of gene *lecB* (Φ coefficient > −0·4). Location is represented as a supplementary variable. 'X'_0 – absence of 'VG'; 'X'_1 – presence of 'VG'; Bor'X' – borehole 'number'; MDep – main deposit; Circ'X' – water circuit 'number'; Equip'X' – equipment 'number'. (a) (+) Centroids *flaG*; (—) Centroids *pilA*; (⋈) Centroids *lecA*; (⧖) Centroids *lecB*; (⬟) Centroids *phzH*; (▯) Centroids *phzS*; (■) Centroids *phzI*; (◇) Centroids *phzII*; (●) Centroids *apr*; (⬬) Centroids *lasB*; (▶) Centroids *exoS*; (▽) Centroids *exoY* and (✱) Centroids Location. (b) (×) Centroids *orfF*; (⬟) Centroids *phzH*; (▬) Centroids *phzM*; (▯) Centroids *phzS*; (■) Centroids *phzI*; (◇) Centroids *phzII*; (●) Centroids *apr* (⬬) Centroids *lasB*; (▶) Centroids *exoS*; (▽) Centroids *exoY* and (✱) Centroids Location.

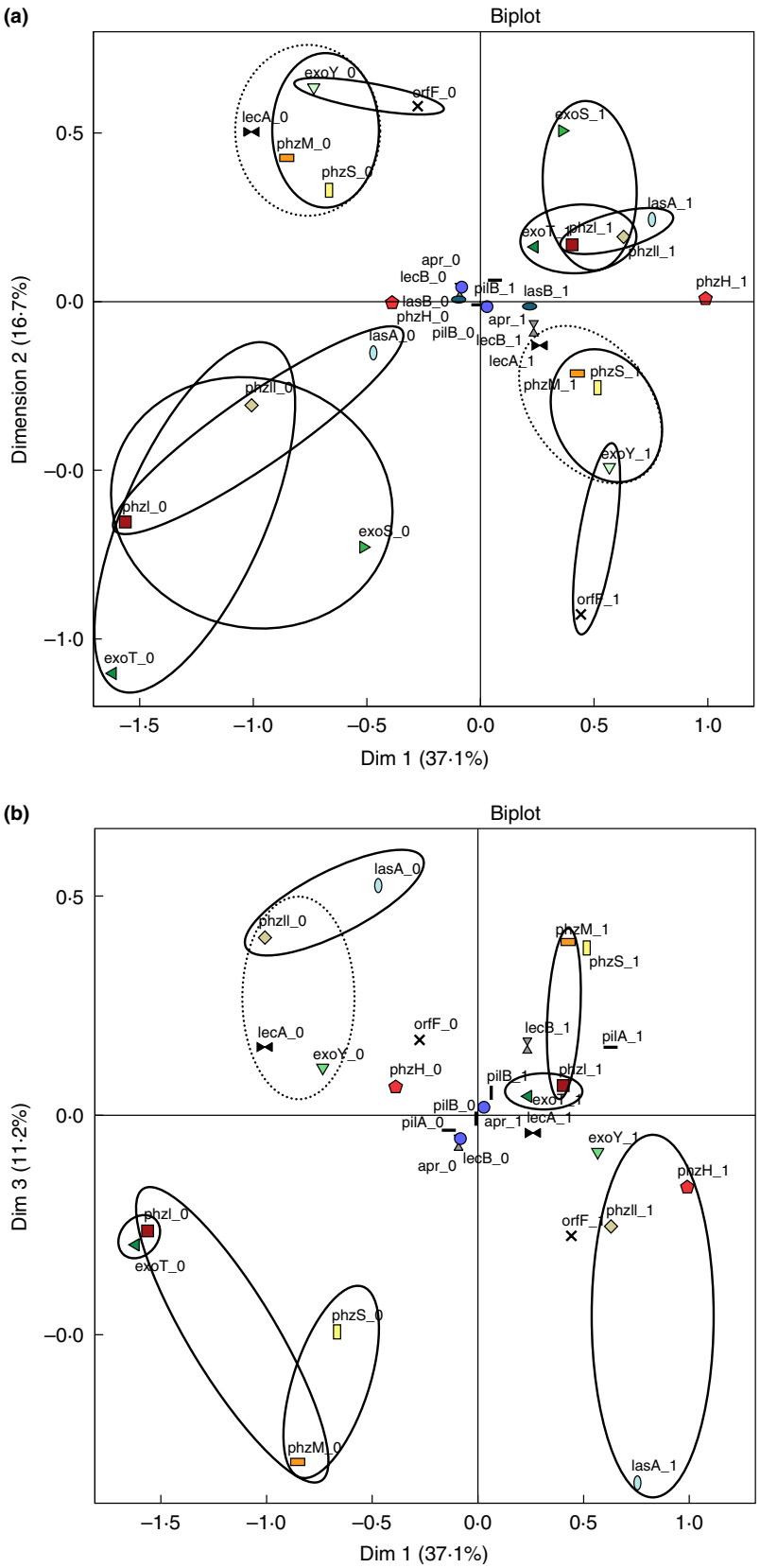


**Figure 3** MCA biplots of the clinical population (a – dimension 1 + 2; b – dimension 1 + 3). In each quadrant, circles highlight relations corresponding to virulence-encoding genes that showed significant association ($P < 0{\cdot}05$). Circles with filled lines represent strong associations ($\Phi$ coefficient > 0·4). Circles with dotted lines represent weak associations (0·2 < $\Phi$ coefficient < 0·4). 'X'_0 – absence of 'VG'; 'X'_1 – presence of 'VG'. (a) (×) Centroids *orfF*; (—) Centroids *pilB*; (⋈) Centroids *lecA*; (⧖) Centroids *lecB*; (⬟) Centroids *phzH*; (▬) Centroids *phzM*; (▮) Centroids *phzS*; (■) Centroids *phzI*; (◆) Centroids *phzII*; (●) Centroids *apr*; (▯) Centroids *lasA*; (⬬) Centroids *lasB*; (▶) Centroids *exoS*; (◀) Centroids *exoT* and (▽) Centroids *exoY*. (b) (×) Centroids *orfF*; (–) Centroids *pilA*; (▯) Centroids *pilB*; (⋈) Centroids *lecA*; (⧖) Centroids *lecB*; (⬟) Centroids *phzH*; (▬) Centroids *phzM*; (▮) Centroids *phzS*; (■) Centroids *phzI*; (◆) Centroids *phzII*; (●) Centroids *apr*; (▯) Centroids *lasA*; (◀) Centroids *exoT* and (▽) Centroids *exoY*.

associated with each other in *thermae* population (Fig. 2), but not in the clinical isolates (Fig. 3). Motility genes did not associate with any genes in clinical population, except *orfF*, strongly associated with *exoY* (Fig. 3). *flaG* and *pilA* genes associated with each other in *thermae* isolates and *pilA* with genes involved in the biosynthesis of phenazines (Fig. 2a). *lasB* and *apr* genes strongly associated with each other and with phenazine and T3SS genes in the *thermae* isolates (Fig. 2). In the clinical isolates, only gene *lasA* revealed association with other genes, *phzH* and *phzM* (Fig. 3b), and no other association was observed. Lectin-encoding genes, *lecA* in *thermae* and *lecB* in clinical isolates, presented weak associations with the genes directing phenazines biosynthesis and to T3SS-encoding genes in both populations (Figs 2b and 3a).

Regarding water system location plots in *thermae* MCA, borehole 2 closely related to the absence of *phzS*, *flaG* and *pilA* genes (Fig. 2a), and equipment 1 and circuit 4 to the absence of *phzH*, *phzS*, *phzII*, *orfF*, *lecA* and *apr* genes (Fig. 2b). Equipment 2 related to the presence of *phzI*, *phzII*, *exoS*, *exoY*, *lasB* and *apr* genes (Fig. 2) and main deposit to the presence of *phzH*, *phzS* and *phzII* genes (Fig. 2a). Borehole 1 and circuit 5 were plotted near negative VG modalities, while circuits 1, 3 and 6 were plotted in the opposite sense (Fig. 2). Circuits 2 and 7 plots were close to the axis and far from VG modalities (Fig. 2), not allowing any assumptions regarding their relation to VG.

### Assessment of acute infection in *Galleria mellonella*

$LD_{50}$s of tested isolates (3 biofilm isolates from equipment 1, equipment 2 and equipment 3, 2 planktonic isolates; 3 respiratory infections, 1 exudate and 1 urine clinical isolates) are present in Table 1. In general, *thermae* isolates presented lower $LD_{50}$s than the clinical isolates (*i.e.* lower bacterial concentrations to initiate infection). Mean $LD_{50}$ of the treatment equipments biofilm isolates (2200 CFU $ml^{-1}$) was similar to the mean $LD_{50}$ of the clinical isolates associated with respiratory infections (2373 CFU $ml^{-1}$), suggesting similar mean ability to cause acute infection. However, differences between $LD_{50}$ of the 3 treatment equipments isolates were significant, ranging from 2× to 10×, implying possible different risks to *thermae* users during treatment. $LD_{50}$s from equipments 1 and 2 isolates were accordant with MCA: B12, from equipment 1, plotted near the absence of phenazine-associated genes plots, presented higher $LD_{50}$ (2×) than B45, from equipment 2, plotted near the presence of phenazine-associated genes plots (Fig. 2). C9, from main deposit, also plotted near the presence of phenazine-related genes plots, presented a low $LD_{50}$, coincidental to the observed for B45. As equipment 3 isolates were not selected for VG study, no relation between its virulence pool and acute infection ability can be addressed.

All clinical isolates presented higher $LD_{50}$s than *thermae* ones, except 003/09. Isolate from exudate presented a $LD_{50}$ 32× higher than the mean $LD_{50}$ of clinical isolates from respiratory infections, which may suggest significant differences in their ability to cause acute infection of isolates that are colonizing different tissues. No information regarding severity or duration of infection upon collection was available.

## Discussion

The outcome of bacterial infections depends on the virulence factors displayed by the bacteria as well as the host response (Sadikot *et al.* 2005). Chronic obstructive pulmonary disease, bronchitis and asthma are considered risk factors for pneumonia, and rheumatoid arthritis and osteoporosis are also risk factors to pneumonia (Vinogradova *et al.* 2009). According to the Portuguese Spas Association (2013), about 25% of the *thermae* users search treatment for respiratory conditions and 50% for rheumatologic conditions, which mean that the majority of the Portuguese *thermae* patients have risk factors associated to pneumonia. Virulence factors are variable traits and their prevalence in different populations should be studied to understand why specific strains colonize and cause infection in specific tissues (Bradbury *et al.* 2010). In present work, we questioned whether the behaviour already described in clinical isolates regarding VG prevalence and infection (Lomholt *et al.* 2001; Hauser 2009; Deptula and Gospodarek 2010) was also observed in the *thermae Ps. aeruginosa* isolates.

In general, *thermae Ps. aeruginosa* isolates presented higher VG rates than clinical isolates, but only 5 VG were statistically significantly more prevalent in this population, with the majority of VG being homogeneously distributed between populations. Authors refer that genes responsible for phenazine biosynthesis, protease and T3SS genes (except *exoU*) are highly conserved among *Ps. aeruginosa* strains from diverse origin, including aquatic ones (Lomholt *et al.* 2001; Bradbury *et al.* 2010), but in present study, this was not observed. Excluding genes involved in motility and adhesion and *exoU*, we observed gene prevalence rates from 94·1% (*exoT*) to 3·9% (*lasA*) in *thermae* isolates and 87·2% (*exoT*) to 28·2% (*phzH*) in clinical isolates. Regarding gene *exoU*, our results were consistent with the literature, for both populations (Lomholt *et al.* 2001; Bradbury *et al.* 2010).

Overall intrapopulation relations of VG observed by MCA highlighted significant differences of the virulence gene global relations in the populations. Some similarity was

observed in the genes involved in phenazines biosynthesis, which was the group with more associations in both populations, although differences were still observed, mainly regarding operon *phzI* in *thermae* population. Phenazines serve as electron shuttles, act as cell signals that regulate gene expression, contribute to biofilm formation and enhance bacterial survival, being extremely important to the cell, apart from their effects on host cells when present in bacteria that are causing infection (Pierson and Pierson 2010). This may explain the high prevalence and associations of these genes in both populations. The associations regarding all other groups of genes were distinct in the populations (motility, protease, T3SS and lectin related genes), suggesting different genetic strategies to adapt to different environments, by means of selective gene loss and regulation. Concerted regulation of VG during infection is well described in the literature (Hauser 2009; Bradbury *et al.* 2010), but to our knowledge, no data are available in the literature regarding environmental isolates, particularly those from *thermae* units. NMW is an oligotrophic environment, where biological availability of nutrients is very low and predation and competition for nutrients very high (Leclerc and Moreau 2002). As virulence factors are natural features of bacteria in order to avoid or survive predation in soil and water (Engel and Balachandran 2009; Hauser 2009), it is not surprising the *thermae* isolates present high VG rates and associations, which might be due to a survival strategy. The majority of $LD_{50}$s in *thermae* isolates were lower than the clinical isolates, which also confirm this assumption.

MCA was also used to study the relations of VG with location in *thermae,* and results indicated that the isolates distributed through the water system and treatment equipments present the behaviour already described in the clinical isolates, where different virulence pool is associated with different sites of infection in the host (Lomholt *et al.* 2001; Hauser 2009; Deptula and Gospodarek 2010). The most significant difference was observed in the treatment equipments, with equipment 1 (collective nebulizer) and equipment 2 (aerosol number 2) plotted in the opposite sense, which was supported by acute infection assays results. From a public health point of view, it is reasonable to consider that the use of different treatment equipments may represent different infection risks to *thermae* users, as biofilm isolates can loosen up during treatment and reach patient's lungs (Pereira *et al.* 2011). Schilliger and Bardelay (1989) presented a case–control study that demonstrated significant association between collective nebulization treatments and pneumonia, of unknown origin, in a French *thermae*, evidencing different infection risks associated with different *thermae* treatments. Coincidentally, the treatment associated with pneumonia in this case–control study was performed with equipment 1-type equipments, which we experimentally determined to have the higher $LD_{50}$ in the *thermae* isolates. We do not know whether the equipment used in the French *thermae* was similar to the one we tested nor the infection agent involved in the study, for which we do not consider the coincidence to contradict our results. Another report from an epidemic of 35 pneumonia and 2 meningitis, also in a French *thermae*, in 1987, does not mention any relation to the treatments that the infected patients used (Hubert *et al.* 1988), as well as Bornstein *et al.* (1989) and Rocha *et al.* (1995), regarding legionellosis, in a French and Portuguese *thermae* report, respectively, being incomplete in that matter.

In summary, the present study demonstrated the high virulence pool of the planktonic and biofilm *Ps. aeruginosa* isolates collected from a *thermae*, with more genetic associations than those observed in the clinical isolates collected from respiratory infections. We also demonstrated the ability of *thermae* isolates to cause acute infection in lower doses than clinical isolates, and that $LD_{50}$ may greatly vary according to location in the water system, suggesting possible different infection risks to *thermae* users according to type of treatments they perform. European regulations do not consider hydrothermal establishments and lack to survey *Ps. aeruginosa* biofilm forms (European Parliament and Council 2009). Epidemiological studies associated with hydrotherapy are rare, incomplete and outdated (Hubert *et al.* 1988; Bornstein *et al.* 1989; Schilliger and Bardelay 1989; Rocha *et al.* 1995). More than 21 million European patients use this natural therapy (Pereira *et al.* 2011), and literature indicates that the majority of *thermae* users present risk factors to pneumonia (Vinogradova *et al.* 2009; Portuguese Spas Association 2013). All this evidences support the need of conducting up-to-date epidemiological studies related to hydrotherapy and possibly a revision of the European regulations that survey the microbiological safety of NMW, to include its use in *thermae* facilities, and particularly to include the survey of treatment equipment's biofilms. We think this would better assure the public health requirements for this important therapeutic alternative, as 'the primary purpose of any rules on NMW should be to protect the health of consumers' (European Parliament and Council 2009).

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## Conflict of Interest

No conflict of interest declared.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1** Statistically significant *P*-values ($P < 0.05$) of chi-square independency tests performed between virulence genes, with respective Phi coefficient values, in the *thermae* and in the clinical isolates.