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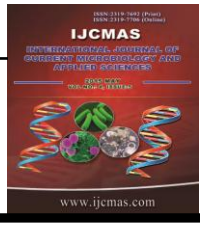

Original Research Article

Susceptibility to ultraviolet light C of *Pseudomonas aeruginosa* biofilms from hydropathic respiratory treatment equipments: impact in water quality control and public health

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Pseudomonas aeruginosa, resistance, chlorine, ultraviolet-C, waterborne infections, natural mineral water

A B S T R A C T

Pseudomonas aeruginosa, a worldwide prevalent pathogen mainly associated to respiratory infections, is ubiquitous in nature, with the water as its preferred habitat. Hydropathic facilities, commonly named *thermae*, are used to treat respiratory health conditions by deep-inhalation of unprocessed natural mineral water. Resistance to commonly used water disinfectants sodium hypochlorite and ultraviolet-C light was tested in *thermae P. aeruginosa* planktonic and respiratory treatment equipments biofilm strains. Half of the isolates resisted to 1% sodium hypochlorite and the other half to 0.5%. Biofilm strains were significantly more sensitive to ultraviolet-C than planktonic forms ($p<0.05$). Low incidence of ultraviolet-C may be a good strategy to disinfect *thermae* respiratory treatment equipments, greatly diminishing the health risk associated to this natural medical practice. With more than twenty-one million users, the majority presenting risk factors for pneumonia, a revision of current hydrotherapy regulations to include this disinfection procedure, without compromising its therapeutic properties, is imperative.

Introduction

Natural mineral water (NMW) is widely used in hydropathic facilities, commonly known as *thermae*. It represents an important therapeutic resource to treat inflammatory respiratory disorders like asthma, bronchitis and bronchiectasis, as well as muscle-skeletal and rheumatologic conditions, among others. To maintain *thermae* NMW therapeutic properties, attributed to its stable physicochemical characteristics, it cannot be disinfected during use, which may pose a risk to the more than 21 million users that annually seek these treatments (Pereira et al. 2014). Thus, the microbiological water quality is a serious concern for *thermae* owners and public health authorities (Costa et al. 2010), particularly when considering the treatments

associated to respiratory conditions. *Thermae*users deep inhale un-disinfected NMW for about 15 to 20 minutes, on a daily basis during 7 to 14 consecutive days, frequently using equipments similar to hospital ventilators (Pereira et al. 2014). There are no directives specifically regulating this natural therapy, leaving each state member the responsibility to legislate, or not, on this practice. The only information available is from the European Council, related to the quality control assessment of NMW, present in the 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 of this pathogen (Ma et al. 2009).Previous studies demonstrated the presence and persistence of *P. aeruginosa* in *thermae* NMW pumping and distribution system, as well as in biofilms of the inner part of respiratory treatment equipments. These isolates showed an increased potential to acquire antimicrobial resistance and similar virulence to clinical isolates from respiratory infections (Pereira et al. 2011; Pereira et al. 2014).

P. aeruginosa is an ubiquitous bacterium, with preference for aquatic habitats, and also an opportunistic pathogen that causes a wide variety of infections, being at present the fifth most prevalent pathogen worldwide (Kanj and Sexton 2012). It is responsible for the majority of the respiratory tract infections occurring in immunocompromised patients, including ventilator-acquired pneumonia and cystic fibrosis (Fricks-Lima et al. 2011), and a significant part of the community acquired respiratory infections (Arancibia et al. 2002).

Folliculitis and otitis are also related to this pathogen, concomitantly associated to the use of aquatic facilities (Mena and Gerba 2009). Its intrinsic resistance to antimicrobials and disinfectants is commonly recognized in the literature (Bak et al. 2010).

Whenever NMW fails to comply with the microbial quality requirements, the *thermae* companies are obliged to apply corrective measures (always performed outside the period of opening to the public) and prove its efficacy to the National public health authorities that regulate this activity in each European state member as soon as possible. A large variety of physical and chemical methods are available for disinfection of water systems, with major emphasis on chemical procedures based on chlorination (Yoder et al. 2008). Ultraviolet-C (UVC) irradiation is another alternative, without generating hazardous byproducts as it happens with chemical disinfections (Costa et al. 2010).

Not granting any disinfectant dispersion to the water obtained with chemicals, which limits its use for the *thermae* water system sanitation procedures, UVC can be useful in the treatment equipments to sanitize the NMW immediately prior to its use. With present work, we intend to study the *thermae* P. aeruginosa isolates resistance to chlorine and UVC disinfection, in order to evaluate the efficacy of current measures applied in the majority of *thermae* and to address the possible use of UVC in these facilities, particularly in the respiratory treatment equipments. They represent the end of the line of the NMW flux from the aquifer to the patient's lungs and can also be contaminated by the *thermae* user itself, deserving a special attention in the sanitation procedures of hydropathic facilities.

Material and Methods

NMW system

The studied Portuguese *thermae* NMW system was composed of 2 boreholes that pumped NMW to a main deposit, from where it was differentially heated and pressurized, before being distributed through 9 circuits that supplied a total of 96 treatment equipments. Pumping and distribution system was made of stainless steel and the majority of the treatment equipments components were made of plastic. Borehole 1 and 2 were distanced from each other in about 50 meters, and about 20 and 50 meters from the main deposit, respectively. The majority of the water distribution circuits were 20 to 40 meters long. The cold water circuit had about 70 meters long and the drinking water circuit 10 meters long. Figure 1 schematically represents the system, with proportional representation of the deposits and therapy pool volumes but not the circuit's length.

Bacterial sample

Water samples were collected on a weekly basis between 2006 and 2009, in a total of 2200 samples. A specimen of each type of treatment equipment (n=11) was fully dismantled and each piece was screened for the presence of *P. aeruginosa*. *P. aeruginosa* planktonic and biofilm isolates were obtained and selected as described elsewhere (Pereira et al. 2011), preserving a total of 226 isolates: 154 planktonic and 72 biofilm isolates from the inner parts of three respiratory treatment equipments. A random proportional sampling of 33% (n=77) of the isolates according to location was performed and fingerprinted by pulsed-field gel electrophoresis (PFGE)toexclude clones (Pereira et al. 2014), retaining a total of 51 isolates: 27 of planktonic origin (9from boreholes and 18 from the water distribution circuits) and 24 biofilm forms from two treatment equipments (15 from equipment 1, 9 from equipment 2),which were used in this study.

Sodium hypochloriteminimal inhibitory concentration

A microdilution assay was performed to determine the minimal inhibitory concentration (MIC) (CLSI 2009) of sodium hypochlorite (SH) in a consecutive 1:2 dilution in Mueller-Hinton (MH)broth (Oxoid, UK), with final concentrations of 4% (v/v), 2% (v/v), 1% (v/v), 0.5% (v/v), 0.125% (v/v) and 0.0625% (v/v). A well containing only MH broth was used as a positive control for each isolate, after inoculation. In each microplate, eight wells scattered through the plate, containing non-inoculated MH broth, were used as negative control. Microplates were incubated at 37ºC, for 24 hours, and results were expressed as growth/no growth, according to evidence of turbidity in each well, measured in the microplate photometer reader Synergy HT. Assays were performed in triplicate.

Resistance to UVC

A 0.5 McFarland suspension, in isotonic saline solution, of each isolate was used to inoculate 3 MH agar plates(CLSI2009). One plate was exposed under 265 nm UVC radiation during 30 seconds and another during 1 minute. The third plate was not exposed to UVC light, serving as positive control for each isolate. Plates were incubated at 37ºC, during 48 hours, and the observed results were classified as: 1 – no growth; 2 – growth in less than half of the interface culture media/platewall; 3 – growth in more than half of the interface culture media/platewall; 4 – growth in less

than half of the culture media surface; 5 – growth in more than half of the culture media surface. Assays were performed in triplicate.

Data analysis

Data were analyzed using SPSS® version 21.0 (IBM) software. Qui-square tests ($p<0.05$) were used to infer to the population the behavior of the sample regarding SH and UVC resistance.

Results and Discussion

Distribution of *P. aeruginosa* in the NMW system and treatment equipments

All planktonic *P. aeruginosa* isolates observed during the 4 years survey were preserved. Their distribution through the water system is described elsewhere (Pereira et al. 2011).

P. aeruginosa was observed in 6 of the 11 sampled treatment equipments: Vichy back massage shower, jet shower, hydromassage bath, collective nebulization and aerosols type 1 and 2 equipments (figure 1), but only isolates from the last three were retained for study (Pereira et al. 2011). Pharyngeal shower, individual nebulization, nasal irrigation, back and full body steam shower equipments did not present *P. aeruginosa* isolates (figure 1).

Resistance to sodium hypochlorite

Minimal inhibitory concentration (MIC) of SH for 24 isolates (47.1%)was 1%, 25 isolates (49%)had a MIC of 0.5% SH and 2 isolates (3.9%)a MIC of 0.25% SH. Table 1 discriminates the SH MICs according to the origin of the isolates: boreholes, water circuits or respiratory treatment equipment biofilms. Two thirds of the isolates collected from bore holes had a MIC of 1% SH, while that was not observed in the isolates from the distribution circuits or from the respiratory treatment equipment biofilms, with around half of those isolates resisting to 1% SH and the other half to 0.5% SH. Two isolates from the treatment equipments revealed a lower ability to resist SH, with a MIC of 0.25% (table 1).Qui-square tests demonstrated no statistically significant association between the isolates type or origin and resistance to SH ($p=0.281$, when considering planktonic vs biofilm isolates; $p=0.276$, when considering borehole vs circuit planktonic isolates).

Resistance to UVC radiation

All *thermae* isolates, except one, were able to grow after 30 seconds UVC exposure but not when exposed to 1 minute UVC radiation. The behavior of *thermae P. aeruginosa* isolates towards 30 seconds UVC radiation greatly differed according to origin: all borehole isolates were classified in the higher classes of growth (3, 4 or 5) and water distribution circuit isolates with similar results, while isolates from the respiratory treatment equipment biofilms only grew at lower rates, with all, except one, being classified in classes 1, 2 or 3 of growth (figure 2), indicative of an inferior ability to recover after UVC aggression. Exposure to 1 minute UVC radiation decreased all isolates ability to grow *in vitro*, particularly planktonic ones, with no isolate classified in class 4 or 5 of growth, as opposite to *in vitro* growth after 30 seconds UVC incidence (figure 2). Qui-square tests inferred to the population the different behavior of the *P. aeruginosa thermae* planktonic and biofilm isolates after 30 seconds UVC exposure ($p<0.001$) or 1 minute UVC exposure ($p=0.017$). When comparing the planktonic isolates according to their origin, boreholes or circuits, qui-square test returned a significant p-value of 0.036 for 48 hours recovery after 30 seconds

UVC exposure. After 1 minute exposure to UVC, no dependency according to origin was observed in the planktonic isolates (p=0.325), highlighting the more expressive difference in the response to different times of UVC exposure by the borehole isolates. More important to emphasize from this work is the fact that biofilm isolates were always significantly more sensitive to UVC light than planktonic ones, irrespective of time of exposure (figure 2).

P. aeruginosa is the major causative agent for foliculittis and otitis in aquatic facilities users (Mena and Gerba 2009) and overall, is the fifth most frequent pathogen worldwide, particularly associated to chronic and acute respiratory infections (Kanj and Sexton 2012). About 75% of the *thermae* users present risk factors for pneumonia, with 25% performing treatments to respiratory conditions (Pereira et al. 2014).

To maintain the microbiological quality standards, *thermae* owners usually perform routine disinfections of the water system, including the one considered in this study, where hyperchlorination of all NMW distribution system is performed on a weekly basis. This strategy, although effective to comply with the Portuguese legislation, as the studied *thermae* was never legally subjected to compulsory closure due to microbiological problems, it is not efficient enough to exempt the system from *P. aeruginosa* (Pereira et al. 2011) or even *Legionella* species (Costa et al. 2010), another important pathogen associated to respiratory infections. Chlorine is the major disinfectant used in water systems (Yoder et al. 2008),due to its easy usage and cost-effectiveness, available as chlorine gas, calcium hypochlorite (solid) or sodium hypochlorite (liquid) (García et al. 2008), with the last one being used in the studied *thermae*. As reported, hyperchlorinationin *thermae* NMW systems takes about 1 to 2 hours and is followed by replacement of the treated water in the system by fresh water (Costa et al. 2010), since *thermae* cannot maintain a chlorine residual,to preserve NMW therapeutic characteristics. These authors also studied the application of UVC light to disinfect NMW system and considered it the most suitable method to control legionellae in *thermae* systems (Costa et al.2010).In present study, we tested *P. aeruginosa* resistance to SH and UVC in order to determine the efficacy of current disinfection measures towards *P. aeruginosa* in the studied *thermae* and to address the possible use of UVC, particularly during treatment, to minimize health risk to*thermae* users.

Chlorine resistance tests provided no statistically significant differences on the behavior of *P. aeruginosa* isolates from diverse origins in the *thermae* system to the presence of different SH concentrations, with isolates resistant to 1% or 0.5% SH being homogeneously scattered through the NMW system and only 2 isolates from treatment equipments biofilms presented a lower MIC of 0.25% SH. *P. aeruginosa* resistance to high chlorine concentrations is documented (Daneshvar et al. 2007). Considering current data, we can anticipate a possible increase in overall *P. aeruginosa* resistance to SH disinfection in *thermae*, as almost half of the studied population already presents higher SH resistance. Rotation in the use of different types of disinfectants in the hospitals is important to minimize acquisition of resistance (Jurgens et al. 2008). We suggest that the same strategy should be implemented in the regular chemical disinfection proceduresof *thermae* and other aquatic facilities, to avoid the rise of chlorine resistance among these isolates, which may compromise even more the efficacy of preventive disinfection interventions.

Table.1 Sodium hypochlorite minimal inhibitory concentration in the thermae *P. aeruginosa* non-clonal isolates

	SH MIC		
	1 % (v/v)	0.5% (v/v)	0.25% (v/v)
Boreholes	6	3	---
Distributioncircuits	8	10	---
Treatmentequipmentbiofilms	10	12	2

SH – sodium hypochorite; MIC – minimal inhibitory concentration

Figure.1 Schematic representation of the natural mineral water pumping and distribution system of the studied Portuguese thermae. A – borehole 1 (35ºC); B – borehole 2 (35ºC); 1 – drinking water circuit (33ºC); 2 – pharyngeal shower circuit (38ºC); 3 – nasal irrigation/aerosol circuit (42ºC); 4 –individual nebulization circuit (55ºC); 5 – therapy pool – 2 sub-circuits (33ºC or 35ºC); 6 – cold water circuit (33ºC); 7 – vapour circuit (60ºC); 8 – hydromassage circuit (38ºC); 9 –Vichy and jet shower circuit (38ºC); Dep. – cold water deposit (33ºC). Yellow arrows indicate the sampled treatment equipments where no P. aeruginosa was observed. Blue arrows indicate those where P. aeruginosa was observed but not preserved. Red arrows indicate the aerosols 1 and 2 (on the right) and the collective nebulizer (on the left) equipments from where P. aeruginosa of biofilm origin was preserved.

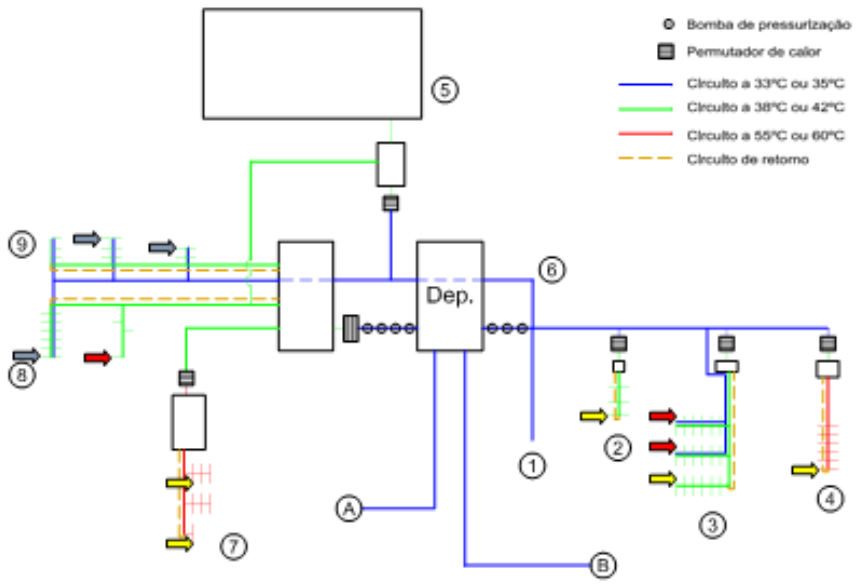
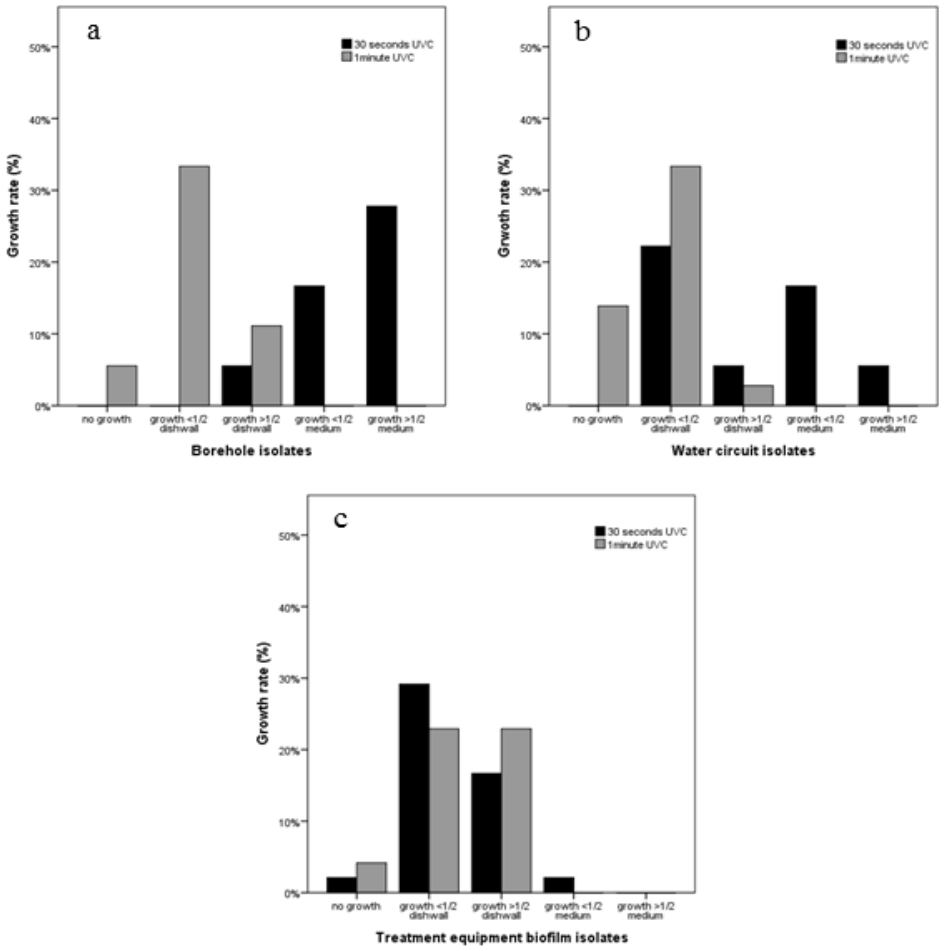


Figure.2 Growth rate (%) at 48 hours incubation of the thermaeP. aeruginosa isolates after 30 seconds and 1 minute UVC radiation. Response to UVC radiation was classified in 5 groups: 1 – no growth; 2 – growth in <1/2 culture medium/plate wall interface; 3 - growth in >1/2 culture medium/plate wall interface; 4 – growth in <1/2 culture medium; 5 – growth in >1/2 culture medium



Regarding UVC irradiation, it was possible to observe a dosage dependent response of the isolates, since the majority of *thermae P. aeruginosa* isolates was able to grow at high rates after 30 seconds UVC irradiation but not after 1 minute exposure. UVC disinfection is very frequent in water circuits (Daneshvar et al. 2007), generating almost no toxic byproducts, contrarily to chemical disinfectants, which make it interesting to use (Hijnen et al. 2006) although no disinfectant residual is possible to obtain (Costa et al. 2010), only ensuring the disinfection of the water locally. UVC is also considered effective to disinfect hospital equipments, but just for a long period of time (Bak et al. 2010). In current study, it was pertinent to observe that the treatment equipment biofilm *P. aeruginosa* isolates were more sensitive to UVC than the planktonic ones, with statistical significance ($p<0.05$). Coincidently, suppliers of *thermae* treatment equipments have recently included a UVC radiation unit in the aerosols, but no information about their efficacy is available. Present results

suggest a minimum of 1 minute UVC radiation of the inner parts of the aerosol equipments to control *P. aeruginosa*. However, we have to highlight that its efficacy varies according to the materials that the equipments are made of and also to the thickness of the biofilms present in them (Bak et al. 2010). Thus, it is important to perform specific tests on these new equipments, in order to evaluate the real efficacy of the UVC units installed on them, testing different materials and different microbial biofilm thicknesses.

Costa et al. (2010) demonstrated that NMW suffered no alteration in the physicochemical parameters during or after UVC irradiation. This means that the NMW physicochemical attributed therapeutic properties are not affected by UVC disinfection, for which we suggest that European countries should legally allow this type of disinfection in their *thermae* facilities, augmenting the microbial safety of this practice without affecting its efficacy. If well implemented, UVC disinfection can surely contribute to a lower health risk in hydropathic therapy, particularly in the treatment equipments associated to respiratory disorders, where higher rates of *P. aeruginosa* in biofilm forms are present (Pereira et al. 2011), with similar virulence to clinical isolates from respiratory infections (Pereira et al. 2014).However, it is important to highlight that this disinfection possibility can bring *thermae* owners to the misleading idea that UVC radiation in the treatment equipments will be enough to assure the NMW microbial quality, and thus eliminating the current preventive disinfection strategies, that are very important to control the microbial growth of biofilms in *thermae* (Costa et al. 2010).

We recently suggested the revision of regulations that survey NMW microbiological safety to include *thermae* facilities, and also to cover the survey of treatment equipment's biofilms (Pereira et al. 2014). We think this strategy, complemented with the permission of UVC irradiation of the treatment equipments will be more adequate "to protect the health of consumers", which is "the primary purpose of any rules on NMW"(European Parliament and Council 2009) than the current weekly survey of the NMW microbiological quality exclusively directed to planktonic forms, performed in some countries. This disinfection approach may represent an important preventive end-of-the-line, usage directed, disinfection strategy, without compromising the natural mineral water therapeutic properties and greatly improving patient's safety and public health.

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