The presence of biofilm forming microorganisms on hydrotherapy equipment and facilities
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ABSTRACT

Hydrotherapy equipment provides a perfect environment for the formation and growth of microbial biofilms. Biofilms may reduce the microbiological cleanliness of hydrotherapy equipment and harbour opportunistic pathogens and pathogenic bacteria. The aims of this study were to investigate the ability of microorganisms that colonize hydrotherapy equipment to form biofilms, and to assess the influence of temperature and nutrients on the rate of biofilm formation. Surface swab samples were collected from the whirlpool baths, inhalation equipment and submerged surfaces of a brine pool at the spa center in Ciechocinek, Poland. We isolated and identified microorganisms from the swab samples and measured their ability to form biofilms. Biofilm formation was observed at a range of temperatures, in both nutrient-deficient and nutrient-rich environments. We isolated and identified microorganisms which are known to form biofilms on medical devices (e.g. Stenotrophomonas maltophilia). All isolates were classified as opportunistic pathogens, which can cause infections in humans with weakened immunity systems. All isolates showed the ability to form biofilms in the laboratory conditions. The potential for biofilm formation was higher in the presence of added nutrients. In addition, the hydrolytic activity of the biofilm was connected with the presence of nutrients.

Key words | balneology, biofilm formation, brine, hydrotherapy equipment

INTRODUCTION

In the natural environment, microorganisms are rarely found as planktonic cells (Mnichowska-Polanowska et al. 2009; Vu et al. 2009; Pantanella et al. 2013), but more often will adhere to surfaces and to each other, creating thin films called biological membranes or biofilms (Czaczky & Myszka 2007; Gajewska & Cieniek 2009; Pietruczuk & Kalicińska 2010; Pantanella et al. 2013; Lecuyer et al. 2015; Otter et al. 2015). A biofilm consists of complex structures in which numerous microbial cells are surrounded by a slime layer (Monds & O’Toole 2009; Pantanella et al. 2013; Lambert et al. 2014). The function of these clusters is to protect microorganisms from the adverse impact of external environmental factors (Ahimou et al. 2007; Lambert et al. 2014; Otter et al. 2015) and to ensure their better access to nutrients (Singh et al. 2006; Vu et al. 2009). Additionally, communication between microorganisms enables them to adapt to changing environmental conditions. As a result, they are able to colonize different ecological niches (Gajewska & Cieniek 2009; Otter et al. 2015), and survive conditions that would be detrimental to planktonic cells (Furowicz et al. 2010).

Pathogens and opportunistic pathogens can colonize biofilms and persist for a long time even under extremely harsh environmental conditions, including high salinity. Whilst embedded in the biofilms, pathogens are protected against stress, chemical agents and disinfectants (Palmer et al. 2007; Tamayo et al. 2010; Xu et al. 2011). Biofilms are difficult to remove and may resist the routine cleaning practices in public facilities such as hotels, hospitals, rehabilitation centers, canteens and other mass caterers as...
well as in private households (Pantanella et al. 2013; Otter et al. 2015). Structures like biofilm can lead to infections and are therefore considered a threat to the safety of hospital patients (Kokare et al. 2009; Pantanella et al. 2013; Lambert et al. 2014; Otter et al. 2015).

Hydrotherapy is the branch of medical science that combines mineral water with modern equipment (for inhalation, underwater and whirlpool massages, etc.) to treat and prevent many diseases and to help patients recover. Due to the relatively high temperature of water (20–38°C) and the availability of nutrients, this equipment provides an ideal environment for the growth and spread of microorganisms, including pathogens (Szczygłowska et al. 2012).

Biofilms adversely affect the microbiological cleanliness of the sanitary installations and hydrotherapy equipment and facilities in spas/hospitals (Otter et al. 2015). In addition, their multi-species structure promotes the growth of pathogens, such as Legionella pneumophila (Leoni et al. 2006) and opportunistic pathogens, for example Pseudomonas aeruginosa (Rice et al. 2012; Leoni et al. 2015; Walker & Moore 2015). Therefore, the aim of this study was to evaluate the ability of microorganisms that colonize hydrotherapy equipment and installations to form biofilms.

MATERIALS AND METHODS

Sampling

Sterile swabs were used to take samples from whirlpool baths, inhalation equipment and submerged surfaces of the brine pool at the spa in Ciechocinek, Poland. The swabs were transported to our laboratory immediately after the collection in insulated containers at ≤7°C.

Isolation and identification of microorganisms

In order to isolate microorganisms, the tips of the swabs were cut off and placed in tubes containing 10 ml of sterile saline. The tubes were then vortexed (for 2 min) to remove microbial cells from the swab material. Ten-fold serial dilutions were prepared from the obtained suspensions using sterile saline diluent. The diluted suspension was then inoculated on to the following media: nutrient agar (composition [g/l]: peptone – 5.00; yeast extract – 3.00; bacteriological agar – 15.00) for strains with high nutritional requirements; and R2 agar (composition [g/l]: peptone – 0.75, yeast extract – 0.50, tryptone – 0.25, glucose – 0.50, starch – 0.50, sodium pyruvate – 0.30, MgSO4 – 0.024, K2HPO4 – 0.30, bacteriological agar – 15.00) for defective strains and strains weakened by physiological shock or with low nutritional requirements. This medium was recommended for the enumeration and cultivation of many bacteria from water. Due to varying nutritional requirements, some strains may grow poorly or fail to grow on this medium (e.g. Legionella pneumophila) (Reasoner & Geldreich 1985).

The two media were prepared using the same concentration of brine (instead of distilled water) that was used in the hydrotherapy equipment we examined (brine pool, whirlpool baths, inhalation equipment). The inoculated plates were incubated at 26°C (as in the investigated equipment) for 7 days.

Isolates were identified using the MALDI method (matrix assisted laser desorption and ionization) accompanied by mass measurement of each sample in a mass spectrometer. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a rapid, reliable, and high-throughput diagnostic tool for the identification of microorganisms (Dingle & Butler 2013). It is based on the ionization of co-crystallized sample material by short laser pulses. MALDI-TOF spectra are used for the identification of microorganisms such as bacteria or fungi (Dingle & Butler-Wu 2015). A portion of a colony of the microbe in question is placed onto the sample target and overlaid with matrix. The mass spectra generated are analyzed by dedicated software and compared with stored profiles. MALDI-TOF MS was carried out with a MALDI Microflex LT (Bruker Daltonics, Bremen, Germany).

Biofilm forming ability of the isolated strains

Biofilm formation by individual strains was quantitatively determined according to Fleming et al. (2009) and Kim et al. (2009).

Start culture

Erlenmeyer flasks, each containing 20 ml of nutrient broth (composition [g/l]: peptone – 5.00, yeast extract – 3.00)
were inoculated with different strains of microorganisms using an inoculation loop, and then incubated for 24 hours at 26°C. Following incubation, the bacterial cultures were diluted 100-fold with saline solution to reach OD<sub>600</sub> = 0.5.

Each strain was tested for biofilm formation in three environments: sterile saline solution (Control); sterile saline solution supplemented with glucose (final concentration 0.25% w/v) (Glucose); and sterile saline solution supplemented with yeast extract (final concentration 0.25% w/v) (Yeast extract).

Subsequently, 200 µl of the prepared suspension (for each variant) was placed in a sterile 24-well microtiter plate. The plates were incubated for 48 hours at 26°C and 30°C. After incubation, all unbound bacteria were removed by rinsing the plates three times using phosphate buffered saline (PBS) buffer (composition [g/l]: NaCl – 8.00, KCl – 0.2; NaH2PO4 – 1.44; KH2PO4 – 0.24) and the biofilm was fixed by drying (1 hour, 60°C). Next, 2 ml of 1% (w/v) crystal violet solution was added to each well and the plates were incubated for 15 min at room temperature. Subsequently, the biofilm was fixed by drying (1 hour, 60°C). The plates were then washed, the dye was solubilized in 0.95% ethanol, and absorbance at 570 nm was determined (Fleming et al. 2009; Kim et al. 2009). Biofilm was classified using the scheme of Stepanovic et al. (2007).

**Evaluation of biofilm hydrolytic activity**

Hydrolytic activity of biofilms was determined using fluorescein diacetate (FDA) (10 mg FDA/ml acetone) (Peeters et al. 2008). Bacterial suspensions were prepared as described above (for the quantitative analysis), and 2 ml of the culture added to each well of sterile 24-well titration plates. The plates were then incubated at 26°C and 30°C for 48 h. After the incubation, the unbound bacteria were rinsed off three times using PBS. Next, each well of the titration plate was filled with 2 ml of sterile PBS and 20 µl of FDA. The plates were incubated in the dark for three hours at 26°C and 30°C. The amount of released fluorescein was measured using a Hitachi spectrofluorimeter F-2500, at an excitation wavelength of 480 nm and an emission wavelength of 505 nm.

**Statistical analysis**

Statistical analyses of variance (ANOVA) with post-hoc Tukey HSD (Honestly Significant Difference) were performed using program STATISTICA 6.0.

**RESULTS**

**Isolation and identification of bacterial strains**

Table 1 shows the results of the identification of the isolated microorganisms. The largest species diversity was recorded among isolates from the whirlpool baths and inhalation equipment, which included several species of gram positive (G+) bacteria, including actinobacteria, gram negative (G−) bacteria, and yeast. The smallest species diversity was observed on the submerged surfaces of the brine pool, which yielded only three species of G+ and G− bacteria and yeast.

**Biofilm formation on titration plates**

All isolates except *Brachybacterium faecium* were shown to form biofilms in the control environment, and in sterile saline supplemented with glucose and yeast extract (Figure 1). In sterile saline, the amount of biofilm measured by absorbance (A) at 570 nm ranged from 0 to 0.137 (Figure 1). The lowest amount of biofilm (A = 0.005) was produced by *Acinetobacter junii* isolated from the whirlpool bath (Figure 1(c)), while the highest (A = 0.137), by *Candida parapsilosis* isolated from the brine pool (Figure 1(a)). According to the criteria described by Stepanovic et al. (2007) biofilms formed by these strains in the uncontaminated environment were weak.

The amount of biofilm was significantly higher (p < 0.05) in saline supplemented with glucose and yeast extract than in the saline solution. The amount of biofilm measured by absorbance (A) at 570 nm ranged from 0 to 0.718 (Figure 1). The smallest amount of biofilm (A = 0.017) was produced by *Microbacterium aurum* isolated from the whirlpool bath (Figure 1(c)). The largest amount was produced by *Stenotrophomonas maltophilia* (A = 0.718) isolated from...
the inhalation equipment (Figure 1(b)). According to Stepanovic et al. (2007), in this case biofilm formation was moderate.

The amount of biofilm produced in saline solution incubated at 30°C ranged from 0 to 0.153 (Figure 2). The highest amount was produced by Micrococcus luteus (A = 0.153) isolated from the inhalation equipment (Figure 2(b)). No biofilm was formed by strains Kocuria spp. and Brachybacterium faecium, isolated from the same equipment (A = 0) (Figure 2(b)). According to Stepanovic biofilm formation was weak.

**Evaluation of biofilm activity**

Hydrolytic activity of biofilms formed by the studied strains in pure saline solution, expressed as the amount of released fluorescein, ranged from 0 to 0.257 µg/ml (Figure 3). The highest value (0.257 µg/ml) was recorded for the biofilm formed by Kocuria spp. isolated from the inhalation equipment (Figure 3b). No hydrolytic activity was recorded for biofilms formed by Brachybacterium faecium and Micrococcus luteus isolated from the inhalation equipment (Figure 3b) and Stenotrophomonas maltophilia isolated from the whirlpool baths (Figure 3c).

In saline supplemented with glucose and yeast extract the hydrolytic activity of biofilms ranged from 0 to 2.521 µg/ml of released fluorescein (Figure 3). The highest hydrolytic activity was recorded for biofilm produced by Microbacterium aurum (2.521 µg/ml), isolated from the whirlpool baths (Figure 3c). No hydrolytic activity was recorded for biofilms formed by Brachybacterium faecium and Micrococcus luteus isolated from the inhalation equipment (Figure 3b) or for Stenotrophomonas maltophilia isolated from the whirlpool baths (Figure 3c).

At 30°C, the hydrolytic activity of biofilms ranged from 0 to 2.446 µg/ml (Figure 4). The highest hydrolytic activity (2.446 µg/ml) was recorded for a biofilm formed by Stenotrophomonas acidaminiphila, isolated from the whirlpool baths (Figure 4c). There was no hydrolytic activity of a biofilm formed by Stenotrophomonas maltophilia, also isolated from the whirlpool baths (Figure 4c).

**Statistical analysis**

The results of the biofilm formation were compared using ANOVA with the Tukey post-hoc test.

For the brine pool, the statistical analysis revealed significant differences between the amount of biofilm formation.
produced in saline with and without the supplements \((p < 0.05)\). At the same time, the results for the control and yeast extract samples were homogeneous according to Tukey’s HSD test. For the inhalation equipment, ANOVA with the Tukey post-hoc test showed significant statistical differences for all samples (control conditions and the culture with glucose and yeast extract) \((p < 0.05)\). The results for the control and culture with glucose were homogeneous according to Tukey’s HSD test. And for the whirlpool baths, the statistical analysis revealed significant differences \((p < 0.05)\), and no homogeneous groups.

**DISCUSSION**

Biofilms on the surfaces of hydrotherapy equipment can be a source of pathogens (Pantanella *et al.* 2013; Otter *et al.* 2015). Moreover, they can interfere with proper functioning of the equipment and facilities (Little & Lee 2007; Gajewska & Cienick 2009; Pantanella *et al.* 2013; Otter *et al.* 2015). This study investigates the presence of biofilm forming strains on a range of hydrotherapy equipment and facilities.

Pathogens, such as *Legionella pneumophila* and *Pseudomonas aeruginosa*, were not identified among the isolated microorganisms; however, opportunistic pathogens
Figure 2 | Biofilm formation on titration plate at 26°C and 30°C for (a) brine pool and its infrastructure, (b) inhalation equipment, and (c) whirlpool baths.

Figure 3 | The hydrolytic activity of biofilm for (a) brine pool and its infrastructure, (b) inhalation equipment, and (c) whirlpool baths.
were identified that may pose a health hazard to spa
patients. The microbiological composition of biofilms
formed on the hydrotherapy equipment depends on the
maintenance of the equipment, contamination in their vicin-
ity, and the users’ health and hygiene standards in the spa
(Capelletti & Moraes 2016). The obtained microbiological
material was very diverse. Among the isolates were gram-
positive and gram-negative bacteria, actinobacteria and
yeast. These microorganisms are common not only in
aquatic environments, but also in the human biota. The
microbial diversity results from a high number of users of
hydrotherapy devices. On their surfaces, patients leave
microbes, parasites and physiological contaminants (epider-
mis, hair, sweat, remnants of cosmetics and cleaning
products, clothing fibers, and dust) (Pasek 2007). The fact
that bacteria indigenous to the human biota were identi-
ified among the isolates indicates their adaptability to conditions
on the equipment used for spa treatments.

The biofilm formation rate depends on its microbial
species composition, physico-chemical parameters, and the
amount and type of nutrients (Ołańczuk-Neyman et al.
2004; Traczewska & Sitarska 2009; van Horn et al. 2011;
Morimatsu et al. 2012). The results of our analysis confirmed
this relationship. A higher amount of biofilm was observed
in saline enriched with glucose and yeast extract than in
pure saline solution. Holà et al. (2006) reported that all the
tested strains showed a better and richer growth of the bio-
film layer at a temperature of 37°C in the nutrient-richer
environment. Similarly, Rochex & Lebeault (2007) found that
increasing nutrient concentration (from 0.1 to 0.5 g l⁻¹
glucose, C/N = 40, C/P = 100) increased the rate and
extent of biofilm accumulation. Lim et al. (2004) demonstra-
ated that increasing glucose concentration to 2.7% in a
given environment leads to an increase in biofilm biomass.
This conclusion has been confirmed by other authors
(Singh et al. 2006; Marić & Vranes 2007). Also Waldrop
et al. (2014) indicated that biofilm mass was increased at
higher glucose concentration. Biofilm mass was increased
at higher glucose concentration with a threshold response
at 0 to 20 and 160 to 200 mg/dL for S. epidermidis and
200 to 240 mg/dL for S. aureus. In turn, limitation of nutri-
ents may regulate the onset of biofilm detachment (Holà
et al. 2006; Morimatsu et al. 2012).

Nyenje et al. (2013) indicated that temperature is yet
another significant factor which influences biofilm for-
mation. The authors demonstrated that E. cloacae can
adhere to surfaces and form a biofilm at 25°C and at 37°C.
Similarly, Zhou et al. (2013) observed biofilm formation by
C. werkmanii at 30°C and at 37°C. Our study confirms this
observation. All examined strains except Brachybacterium
faecium formed biofilms, both at 26°C and 30°C. A small
temperature difference did not cause significant differences
in the amount of biofilm. Di Bonaventura et al. (2007)
showed that 32°C was optimal temperature for biofilm for-
mation by Stenotrophomonas maltophilia, while Holà
et al. (2006) found that 37°C is the most favorable for this
species. Iversen et al. (2004) evaluated microbial adhesion
at high temperatures (>40°C) and found that the ability to
form a biofilm within this temperature range (26–40°C) is
associated with the optimum growth temperature of

Figure 4 | The hydrolytic activity of biofilm at 26°C and 30°C for (a) brine pool and its infrastructure, (b) inhalation equipment, and (c) whirlpool baths.
different microbial species. At the optimal temperature for growth cell numbers increase rapidly. This facilitates sedimentation, which accelerates the initial adhesion and the formation of microbial biofilm (Marinho et al. 2013). Our study was conducted at temperatures similar to those that are selected for spa treatments using the examined hydrotherapy equipment.

Crystal violet staining is widely used for determining the amount of biofilm formed by a wide range of microorganisms (Stepanovic et al. 2007); however, the method does not produce consistent results (Peeters et al. 2008). Moreover, it is poorly suited for studying the viability of biofilm cells as crystal violet stains both bacterial cells (alive and dead) and the biofilm matrix (Peeters et al. 2008). The alternative method using FDA has gained popularity in the evaluation of the viability and the activity of biofilm cells. Simple and efficient, the method guarantees high repeatability of results and is therefore considered highly reliable for the quantification of biofilm (Peeters et al. 2008).

**CONCLUSIONS**

The study confirmed the possibility of biofilm formation on the surfaces of hydrotherapy equipment. The isolated and identified microorganisms were very diverse. No common pathogens were identified among the isolates. All isolates were classified as opportunistic pathogen which can cause infections in humans with weakened immunity systems. All isolates showed the ability to form biofilms in the laboratory conditions. Biofilm formation was observed at both 26°C and 30°C. The introduction of nutrients (to simulate contamination) increased biofilm formation. This indicates that the contamination of natural raw materials (brine) and hydrotherapy equipment enhances biofilm formation and may lead to serious infections. For this reason, it is necessary to regularly monitor equipment, facilities and installations and also water use in spas.

**REFERENCES**


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