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BRIEF COMMUNICATION

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Detection and characterization of *Bacillus cereus* isolated from the dialysis fluid

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ABSTRACT

In this study, *B. cereus* was detected in dialysis fluids within international parameters (ultrapure – maximum limit of 0.1 CFU/mL for heterotrophic bacteria count) by analyzing the pellet obtained through the centrifugation method. We also investigated the ability of the *B. cereus* isolate to form a biofilm at different temperatures, the production of virulence factors, and the susceptibility to commercial antimicrobial agents. This study demonstrated a high ability of *B. cereus* to persist in the hemodialysis system, which can be explained by its broad ability to produce a biofilm at 25 °C, its relevant production of virulence factors, such as β-hemolysin, lecithinase and cereulide, and its important resistance pattern to antimicrobial drugs. In conclusion, these new findings expand the understanding that this microorganism should not be neglected and new methods for tracking it should be considered.

KEYWORDS: Renal dialysis. Centrifuge. *Bacillus cereus* group. Biofilm.

INTRODUCTION

Within renal replacement therapy, hemodialysis, is one of the most important treatments for patients with chronic renal failure[1]. Despite the numerous barriers present in the treatment system to remove microorganisms from the water, there is a risk of contamination if the system is not efficient[2]. In addition, contamination by a biofilm-forming microorganism can persist at various points along the treatment system and increase the resistance to disinfection and antimicrobial procedures, leading to endotoxemia and/or infection[3]. The aim of this study was to detect, identify and analyze the conditions of virulence, biofilm formation and susceptibility profile to antimicrobial agents of *Bacillus cereus*.

MATERIALS AND METHODS

Dialysis fluids were collected from 8 sites of the hemodialysis (HD) circuit. The collections were performed in a hemodialysis clinic of a hospital located in the Northwestern region of Alto Jacui, Rio Grande do Sul State (RS), Brazil, during the months of September, October, and November 2018, according to the guidelines of the American Public Health Association[4]. After the collection, transport and homogenization, the samples were subjected to a centrifugation process according to Paterson, but with some adaptations[5]. Gram stains and the presence of spores were examined[4]. For biochemical analysis, a bacterial suspension was added to the different wells of the API 50CHB and the exact molecular masses

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of the components were determined using the MALDI-TOF. The DNA was extracted from the colonies using the cetyltrimethylammonium bromide technique, according to Moore *et al*.[6].

The β-hemolysin production and the lecithinase and cereulide detection were performed in accordance with Fritze[7]. For biofilm formation, the assay was performed in 96-well plates, as described by Bonez *et al*.[8]. The plates were incubated at 25 °C (dialysis water circuit temperature), 37 °C (dialysate temperature), and 46 °C (temperature associated with toxin production) for 24/48h to promote microbial adhesion.

The biofilm formation was assessed using the crystal violet (CV) technique in order to estimate the total biomass, as described by Bonez *et al*.[8], but with some modifications. Three-dimensional analysis of the structure of the biofilm formed by *B. cereus* on high-density polyethylene (HDPE) sheets was observed using atomic force microscopy (AFM) according to the method of Quatrin *et al*.[9] with modifications.

The antimicrobial susceptibility testing was performed using the disk diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute[10], but with modifications.

Data are presented as mean ± standard deviation using one or two-way analysis of variance (ANOVA), as appropriate, followed by the Tukey's test. The analyzes were performed using GraphPad Prism software version 6.01 for Windows (GraphPad Software, San Diego, CA, USA). Normality was confirmed with the Shapiro–Wilk' test and the correction of hypothesis test values was carried out using the Bonferroni' method. Significant data are reported as mean differences (MD) and their respective confidence intervals (95% CI). P values < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

In this study, *B. cereus* was detected in the dialysis fluid, within international parameters (ultra-pure – maximum limit of 0.1 CFU/mL for counting heterotrophic bacteria) by analyzing the pellet obtained through the centrifugation method. Countries such as the United States and Japan have expressed concern about the incidence of bacteremia in patients undergoing HD. This has stimulated process improvement measures to prevent chronic inflammation in these patients and possible infections due to dialysate contamination[11].

In this analysis, a growth of more than 100 CFU/mL was detected in the blood agar plates of the dialysis fluids from two HD machines and also in the reuse solution. In the prefilter, in the supply line of the HD unit, in the pre-osmosis and in the dialysis water of 3 HD machines, < 100 CFU/mL were found, identifying the microorganism of the *Bacillus cereus* group.

The identification of the microorganism Gram stain (Gram-positive), the presence of spores (positive) and the formation of subterminal oval spores (sometimes cylindrical) through the API method and by using commercial database for MALDI-TOF proved to be inconclusive for the *B. cereus* species (score of 2.039), which is considered consistent with an accurate identification at genus level and probable identification at species level. Subsequently, the consensus sequence was subjected to BLAST analysis in order to compare it with the sequences deposited in the GenBank. This revealed that the isolate identified in HD was 99.92% consistent with the sequences of *Bacillus cereus* (strains T-o4 [KY852255.1] and JT -86 [MW889122.1]) and 99.85% consistent with *Bacillus thuringiensis* (strain Bt-GS57, CP043234.1). The need for dialysis fluid purification, the impact of the water treatment and the distribution system on water quality, as well as the dialysis machine itself, which represents a potential source of contamination, have been discussed since the 1980s[11].

Within 24h, the microorganism produced strong β-hemolysis in the blood agar plates, and the incubation was extended to 48h, which increased the detection of β-hemolysis. The presence of lecithinase was detected on agar with egg yolk extract by the formation of an opaque zone around the colony on agar. In the starch hydrolysis assay, a light zone was observed around the colony on nutrient agar with added starch, indicating the production of cereulide (Figure 1).

Some time ago, *B. cereus* was generally considered a contaminant when isolated from various sources (blood, wounds, exudates, and saliva)[12] due to its widespread germination in soil, dust, water, and hospital environments. However, over time, it gained notoriety due to nosocomial outbreaks in hospitalized and immunocompromised patients with hematologic neoplasms and chronic diseases, including end-stage renal disease, typically associated with a lower level of immunosuppression[13].

The *B. cereus* isolate showed the ability to form a biofilm after 24h of incubation. Different temperature conditions (25 °C, 37 °C, and 46 °C) were tested and significant biofilm biomass formation was detected at 25 °C (Figure 2). This may indicate adhesion of the biofilm in the pipeline, through which the HD fluids are transported, when associated with its high resistance to biocides and its presence, even when the parameters are within the established limits, confirming the results, which indicated that this represents an important

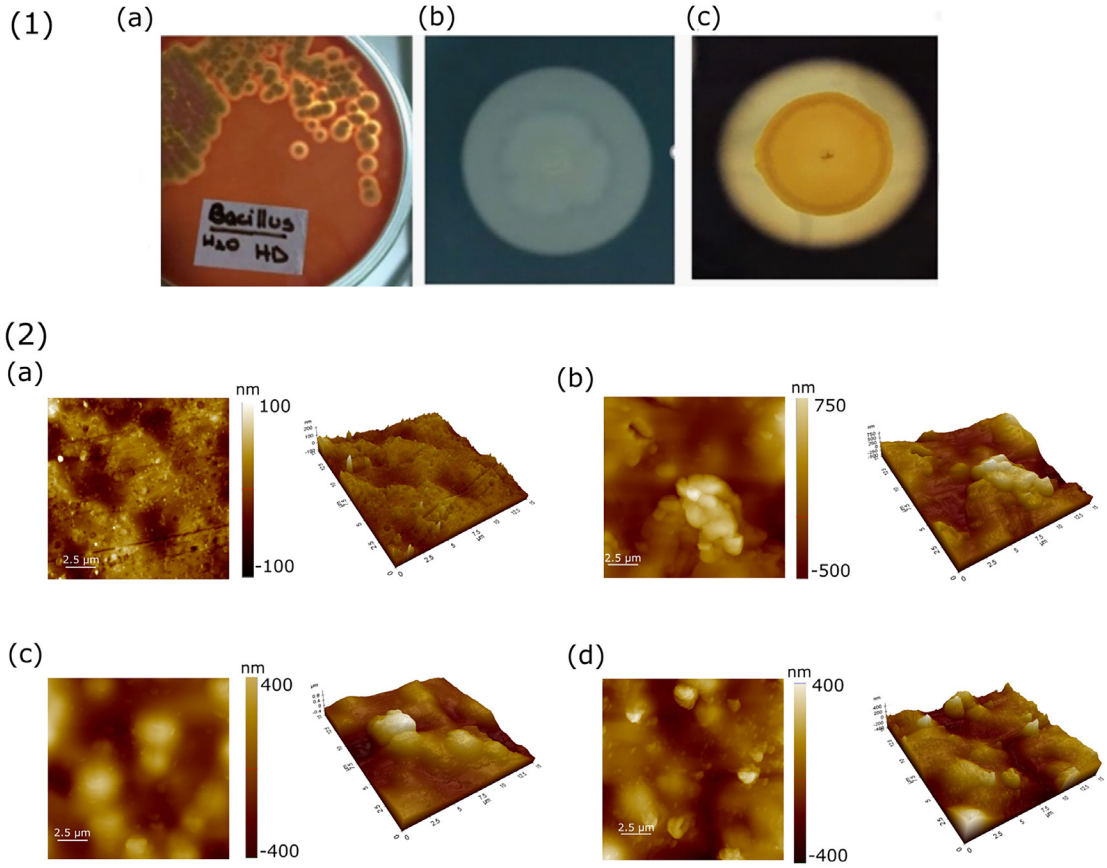

Figure 1 - 1) Representation of the bacterial isolate in culture media for detecting different toxins: a) blood agar morphology of the *B. cereus* colony grown in blood agar plate under aerobic conditions at 36 °C and surrounded by a clear zone, indicating hemolysis; b) Trypticase soy agar enriched with egg yolk, on which an opaque zone around the colony indicates the presence of lecithinase; c) Nutrient agar plus starch, in which the formation of a halo around the colony indicates the presence of cereulide; 2) Topographic AFM images of the biofilms formed from *Bacillus cereus* at different temperatures over a total scan area of 15 µm^2: a) Negative control; b) 25 °C; c) 37 °C; d) 46 °C.

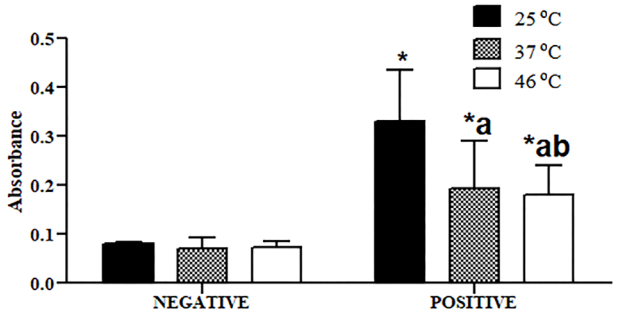

Figure 2 - Biofilm quantification of *B. cereus* isolated from the hemodialysis water at 25 °C, 37 °C, and 46 °C using the crystal violet technique. Data are presented as mean ± standard deviation. $p<0.05$(*) were considered statistically significant when compared to the negative control. $p<0.05$ (a) compared to 25 °C, $p<0.05$ (b) and compared to 37 °C. The threshold indicated by a solid bar is equal to the background signal plus three times the standard deviation (OD = 0.1). Values higher than the threshold values were considered positive for biofilm formation.

mortality risk factor[14,15]. The quantification test allowed for the classification of the strain into strong adherence (+++) at 25 °C corresponding to the dialysis circuit, and weak adherence (+) at 37 °C (in the dialysis machine) and at 46 °C. In fact, a difference in topography can be observed in three-dimensional images, confirmed by the obtained roughness value (Ra), which confirms the increase in biofilm formation at 25 °C, where Ra was 151.89 nm, whilst the negative control showed only 26.186nm (Figure 2).

The temperature of 46 °C was tested because heat disinfection is currently being recommended, but its dose is not clearly defined in literature reviews[16,17]. When heat is combined with chemical detergents, it shows better efficacy in reducing CFU, but it is still unable to completely eliminate the biofilm[18].

These results suggest that this microorganism has the ability for strong biofilm adhesion in the dialysis circuit and this may be associated with resistance to biocides and antimicrobials, even within the normal parameters of the dialysis fluid quality. It is worth noting that bacteria growing in a biofilm state are thousands of times more tolerant to antimicrobial measures than in a planktonic state[15].

In clinical settings, the development of biofilms by *B. cereus* can lead to infection due to the persistence of bacteria in medical devices, thus serving as a reservoir for disease[18,19]. Biofilm strains of *B. cereus* have been reported to cause nosocomial bacteremia following catheter-related infections[17] and have the potential to cause catheter-related bloodstream infections even in immunocompetent patients[13]. Studies suggest[13,18] that a more detailed investigation of the *B. cereus* group is needed, including molecular aspects involving genes in order to clarify their distribution. They cannot be considered simple contaminants, especially in hospital settings.

The susceptibility of *B. cereus* to antimicrobials was tested against 22 selected antimicrobials. The natural resistance to β-lactams was confirmed, with resistance to the beta-lactamase inhibitor piperacillin + tazobactam, trimethoprim-sulfamethoxazole, and aztreonam. The isolate was found to be sensitive to rifampicin, erythromycin, clindamycin, ciprofloxacin, chloramphenicol, gentamicin, tetracycline, and vancomycin.

Effective antibiotic therapy is considered the most important treatment to eradicate *B. cereus* infections[20], so it was necessary to investigate the susceptibility of the microorganism to antimicrobial agents. In this study, the resistance to β-lactams was detected, although the isolate showed a sensitivity to quinolones, aminoglycosides, and macrolides, which is consistent with previous studies[13]. In particular, vancomycin, ciprofloxacin, clindamycin, imipenem and aminoglycosides were treatment options for diseases caused by *B. cereus*[20].

Safe dialysis requires careful temperature regulation, concentration, flow rate, and pressure, as well as adequate disinfection[17]. However, all water systems are prone to unmonitored failures, even in modern systems that use more technologically advanced equipment[16].

Ensuring and keeping the quality of the dialysis fluid used in HD procedures is critical to maintaining the quality of life of chronic kidney disease (CKD) patients. Patients with CKD are immunocompromised, which puts them at higher risk of acquiring infections than other people. Infections are a major cause of mortality and morbidity among these patients[12].

CONCLUSION

Based on the findings herein, new research should address water and dialysis fluid monitoring and the routine testing of analytical methods as well as disinfection procedures, including biofilm formation monitoring should be strengthened. This should comprise the investigation of the occurrence of adverse events related to contamination problems and the correlation between water contamination and the condition of treated patients, in order to guide hemodialysis treatment in new directions.

It must be considered that the microbiological quality of the dialysis fluid does not depend only on reverse osmosis, but on all the systems of maintenance, disinfection, and implementation, as well as on the constant renewal of prevention protocols. This study proved the high persistence capacity of microorganisms of the *B. cereus* group in the dialysis circuit, with biofilm production and the release of toxins. Therefore, our findings expand the understanding of how this microorganism cannot be neglected. Thus, we expect that this study can contribute to future research that will complement the safety and quality of life of patients in need of renal replacement therapy.

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