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STUDY OF BACTERIAL PROLIFERATION USING A METHOD THAT SHOWS BACTERIAL GROWTH DEPENDING ON THE HEAT RELEASED – MICROCALORIMETRY

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Infectious diseases are important issues in any healthcare system of any country in the world. Given the increased morbidity and mortality related to infections associated with the medical procedures, it is necessary to develop rapid and effective diagnostic methods. One method that has managed to gain ground in the age of technological evolution is microcalorimetry. It can be used successfully today, with a very high sensitivity, detecting heat flow changes in the order of micro/nano-joules. Bacterial microcalorimetry has a number of advantages and as such should be considered as a possible method of rapid and effective diagnosis. For this study we used microcalorimetry to evaluate the *S. aureus* and *K. pneumoniae growth curves*, but the technique can be applied to any pathogen. In order to use this method in clinical studies, numerous experiments are needed to be performed, following the impact of as many parameters as possible, in order simulate the clinical conditions. Once sufficient stackable experiments are cumulated, by corroborating them, a working algorithm can be created with the ability to provide information with high sensitivity.

Keywords:

EPIDEMIOLOGICAL DATA RELATED TO INFECTIOUS IN THE FIELD OF ORTHOPEDICS AND TRAUMATOLOGY

Orthopaedics and traumatology is constantly developing, integrally connected to technological progress, in conjunction with other medical specialities. Infectious disease is a major problem in health systems. In the case of orthopedics, but also of other surgical specialties, the infection of a wound translates into slowing down the healing process accompanied by an increase in the psychological stress suffered by the patient. In these cases, patients describe increased pain compared to those who, having the same pathology, were cured without associated infection[1,2]. The correct and rapid diagnosis followed by an adequate treatment is essential in this type of pathology, because the infection of a surgical wound, especially in the case of arthroplasties or the use of orthopedic implants, can produce a disastrous effect in most cases requiring the removal of implanted material[3,4].

This paper will present microcalorimetric experiments performed on two lyophilized germs. The purpose is to present the ability of the microcalorimeter to produce superimposable bacterial growth curves, and in order to be easy to follow.

All postoperative infectious processes interrupt the normal healing process causing an increased risk of associated pathologies or decompensation of pre-existing ones, but at the same time prolong the patient's hospitalization time causing additional costs on health systems[5,6].

The first process is the coating of the prosthetic material by serum proteins and platelets, these proteins facilitate the adhesion of pathogens through different receptors. These pathogens gradually multiply, forming the biofilm with the ability to resist the host's cellular and humoral response[7].

Given the increased morbidity and mortality related to the situations in which infections are associated with the medical procedures, it is necessary to develop rapid and effective diagnostic methods. The speed of obtaining a positive, accurate diagnosis is important because in the case of the orthopedic field, but not only, the targeted treatment can save a limb and can restore the mobility of a patient with few resources[8].

MICROCALORIMETRY

One method that has managed to gain ground in the age of technological evolution is microcalorimetry. It can be used successfully today, with a very high sensitivity, detecting temperature changes of the order of micro/nano-joules. The ability of bacteria to release heat through metabolic activity is the foundation of the present study, the microcalorimeter being able to record the energy released due to exothermic biochemical reactions[9,10].

The use of a liquid sample, inoculated with an organism, which will provide a growth curve, is a method by which calorimetry describes bacterial growth and by which this method can be used for bacterial identification[11,12]. One of the experimental parameters that we can easily change is the culture medium, which significantly influences the bacterial growth without changing the growth curve model. The possibility of knowing the parameters inside the sample, such as the amount of CO_2, oxygen concentration or pH, gives us an overview of what is happening inside the calorimeter cell, which facilitates the identification of the infectious agent[13,14].

Bacterial microcalorimetry has a number of advantages and as such should be considered as a possible method of rapid and effective diagnosis. Sensitivity is an important asset. Microcalorimetric signs of bacterial multiplication can be detected; even when as little as 10,000–100,000 active bacterial cells in cultures are present. In addition to highlighting the presence of organisms, the generated curve has features that facilitate the identification of the pathogen involved. It is also worth noting that this method gives us real-time information: we can check the evolution of bacterial growth at any time, through a computer or even remotely, using a smart phone. In addition to the ease and speed of data processing, when we make a microcalorimetric experiment we can juggle an increased number of variables (temperature, culture medium, recording method) that correlate with information already known in the literature that offers us multiple possibilities diagnosis and subsequent "targeted" treatment[15,16].

The ability of microcalorimetry to record thermal energy was demonstrated in a study that compared the time required for a bacterial culture to be detected at different concentrations of inoculated pathogen. The study used different bacterial strains (*Candida albicans*, *Escherichia coli*, *Staphylococcus aureus* and *S. epidermidis*, *Streptococcus sanguinis*, *Cutibacterium acnes*) which were inoculated in liquid medium and left in the microcalorimeter for 5 days at a constant temperature of 35º C. Bacterial growth curves were specific to each species, the disposition pattern was independent of the number of organisms with which the experiment was started, and a heat released over 10μW from the baseline was accepted as a positive detection. Experiments with an initial bacterial load of 10^5 CFU / mL identified *E. coli*, *S. aureus* and *S. sanguis* in less than 4 hours. The pathogen load of the microcalorimetric cell must be reduced in studies with fungus because the dimensions of the fungi are significantly bigger than those of the bacteria, and an overload of the microcalorimetric cell produces erroneous information. For experiments with *C. albicans* the amount of organisms was reduced (10 CFU / mL) and 43 hours were required to detect it[17,18].

We live in a world in danger because of microorganism resistance to antibiotics and chemotherapeutics, in a world where "resistance" has started and evolved towards "multi-resistance", towards "extended resistance" and even towards "total resistance". In a world where the danger of increasing the resistance of microorganisms to antibiotics and chemotherapeutics is discussed at the highest level (political, administrative, medical), any method that facilitates targeted treatment must be supported and developed. Differential isothermal microcalorimetry allows us to analyse bacterial growth in the absence or presence of compounds with antimicrobial capabilities, this new method can be introduced into the medical diagnostic arsenal after its standardization[19,20].

In addition to methods such as serial dilution of antibiotics in liquid media, quantitative determinations by E-test, or the use of antibiotic-impregnated discs after seeding the bacteria on the plate, microcalorimetry provides information on throughout the experiments and not just at the end of them. The studies by Higuera-Guisset *et al.* in 2005 showed that a single bacterium can produce

heat of ~ 2 pW; sowe can detect the approximate number of bacteria at any time in the experiment[21].

THE UTILITY OF MICROCALORIMETRY IN OTHER FIELDS

Microcalorimetry can also be used in many other fields. In a study designed to improve the environment, microcalorimetry was used to highlight the effects of some substances on it. Using a population of *Bacillus subtilis*, the harmful effect on the aquatic environment of harmful substances such as hydroquinone, catechol or resorcinol was studied. Another utility of this type of study was the development of methods that, through the process of bacterial biodegradation, can purify an environment with microbial enzymes, provided that the organisms in question can grow in the environment used[22].

In 2009, a team of researchers tested the ability of *Acinetobacter* spp. and *Pseudomonas* spp. colonies to process benzene and toluene using microcalorimetry. These organisms had these organic solvents as the only source of nutrients and managed to significantly degrade them (between 67% and 94%) in about 72 hours. This phenomenon was highlighted with the help of microcalorimetry, the bacteria developing growth curves in the conditions in which the experiments were performed with increasing solvent concentrations from 1% to 70% of the microcalorimetric cell volume[23].

In another study conducted in 2010 by Chen *et al.* it has been shown that the rate of inhibition of bacterial growth can be calculated depending on the concentration of harmful substances in the environment, processing by logarithmic microcalorimetric curves. These data can be used in effect prediction equations that provided relevant information, insignificantly different from the data obtained from the experiments, objectifying the ability to predict microcalorimetry[24].

THE ROLE OF SONICATION IN OPTIMIZING MICROBIOLOGICAL DIAGNOSIS

In orthopedics, for the evaluation of an implant infection, it has been shown that intraoperative tissue sampling has a sensitivity of 93%, higher compared to sampling the pathological product and seeding it by classical methods represented by joint aspiration or swab harvesting from an active fistula, when the sensitivity reaches a value of about 70%[25]. The ability of bacteria to develop biofilm is known, which gives them resistance to antibiotic treatment and the host immune system[7,26].

The generation of biofilm, both at the interface with the bone and at the interface with the implanted material, is also a problem in establishing the correct diagnosis. A modern method that comes to the aid of the attending physician is sonication, which manages to mobilize the bacteria in the biofilm, so that they can be subsequently isolated. Using an ultrasound-producing device, microbial agents can be extracted from the sample collected from the infected area, thus increasing the sensitivity from 73% to 91% and the specificity from 93% to 97%, as demonstrated by Rak or Sampedro *et al.*[25,27,28].

In order to make a correct diagnosis, both Tunney[29] and Trampuz[30] together with their teams demonstrated that the use of sonication for the detachment of the biofilm adherent to the implant is a method that increases the identification rate of the microbial agent.

MATERIALS AND METHODS

EXPERIMENTS PERFORMED TO DEMONSTRATE THE REPRODUCIBILITY OF MICROCALORIMETRY

In the perspective of conducting research on single-channel microcalorimeters, experimental reproducibility is essential. Without demonstrating reproducibility and highlighting the factors influencing it, some conclusions regarding comparisons between experiments performed under various experimental conditions are not sufficiently supported. The main problem of bacterial microcalorimetry performed on single-channel apparatus is that the samples (bacterial cultures) must be freshly prepared, and have a well-described bacterial concentration (it is known that the bacterial concentration influences the latency time) and be introduced as faster in the calorimeter after preparation, because these cultures become metabolically active at room temperature and the delay in performing the thermal analysis can escape the first stage of growth.

For the experiments we used the following organisms *Staphylococcus aureus* – ATCC 25923

(Fig. 1) and *Klebsiella pneumonia* ATCC 700603 (Fig. 2). The media were trypticase soy agar (TSA) and Mueller-Hinton broth (MHB). The experiments performed at 37º C; with a loading volume of microcalorimetric cells of 600 µL are presented in the figures below.

WORKING PROTOCOL

In order to perform experiments with increased reproducibility, the stages of preparation of the experiments must be performed in the same way each time. The first step in developing an experiment is to prepare a liquid culture with the infectious agent to be investigated. These cultures must be freshly prepared for each experiment, because over time the number of non-viable bacteria accumulates in the investigated environment can be misleading by an increased artificial nephelometric index. The second necessary step is to prepare the samples and introduce them into the microcalorimeter as soon as possible because the bacteria used have the ability to grow at room temperature. If an experiment is initiated after a long time from the preparation of the sample, the initial growth stage may be lost. Another very important aspect when working with a microcalorimeter is to maintain a constant ambient temperature, because the appearance of temperature fluctuations in the room can cause artifacts on the bacterial growth curve. Taking into account the above, we can conclude that the development of a working protocol and rules of conduct in the room for microcalorimetry are essential for successful experiments.

RESULTS

In order to correctly evaluate the growth curves we proposed some useful parameteres, namely: the isoelectric baseline at the beginning of the experiment and the return to this line at its end, the maximum growth peak reached by the bacterium, thermal signal detection and exponential growth. Using these parameteres we can characterize raw thermograms of bacterial growth or we can differentiate the organisms.

We evaluated the growth of *S. aureus* and *K. pneumoniae*; the techniques could be use for different organisms. As presented in the adjacent figures the time interval after which we can identify a growth curve, does not exceed 15–20 hours.

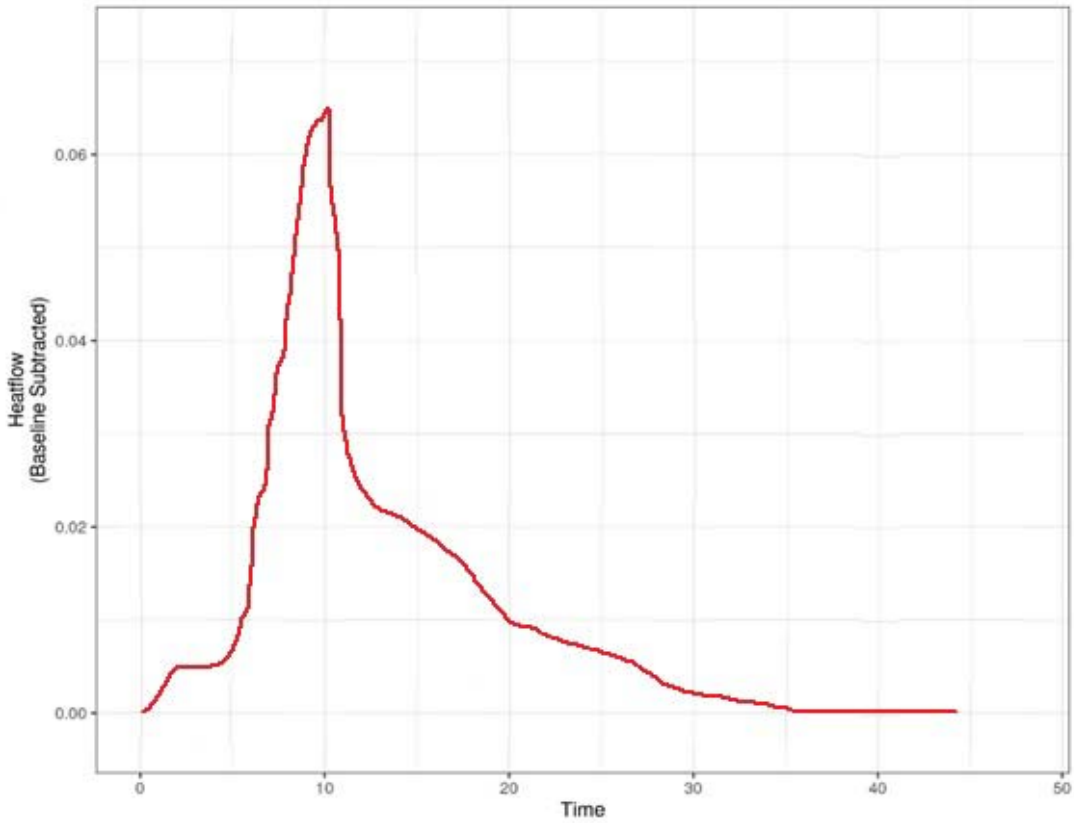

Figure 1. Graphical representation of the growth curve generated by the heat released by the active metabolism of *S. aureus*. Experiments performed at 37º C, with a microcalorimetric cell load of 600 µL.

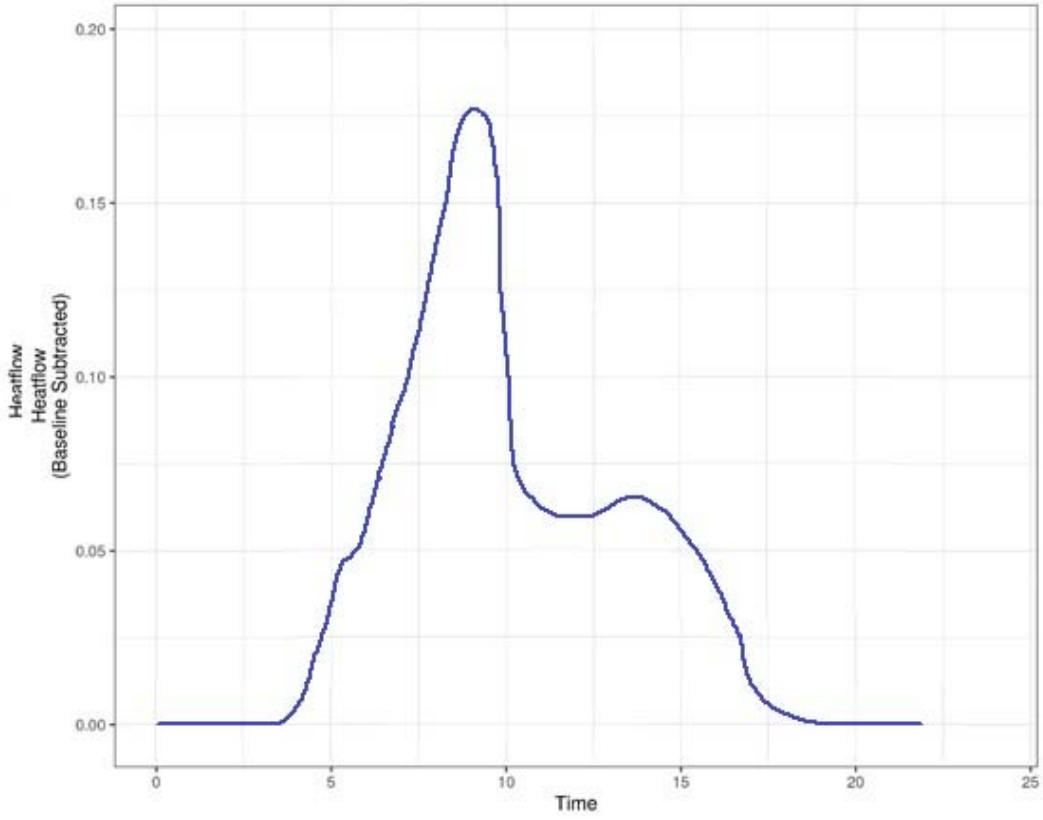

Figure 2. Graphical representation of the growth curve generated by the heat released by the active metabolism of *K. pneumoniae*. Experiments performed at 37º C, with a microcalorimetric cell load of 600 µL.

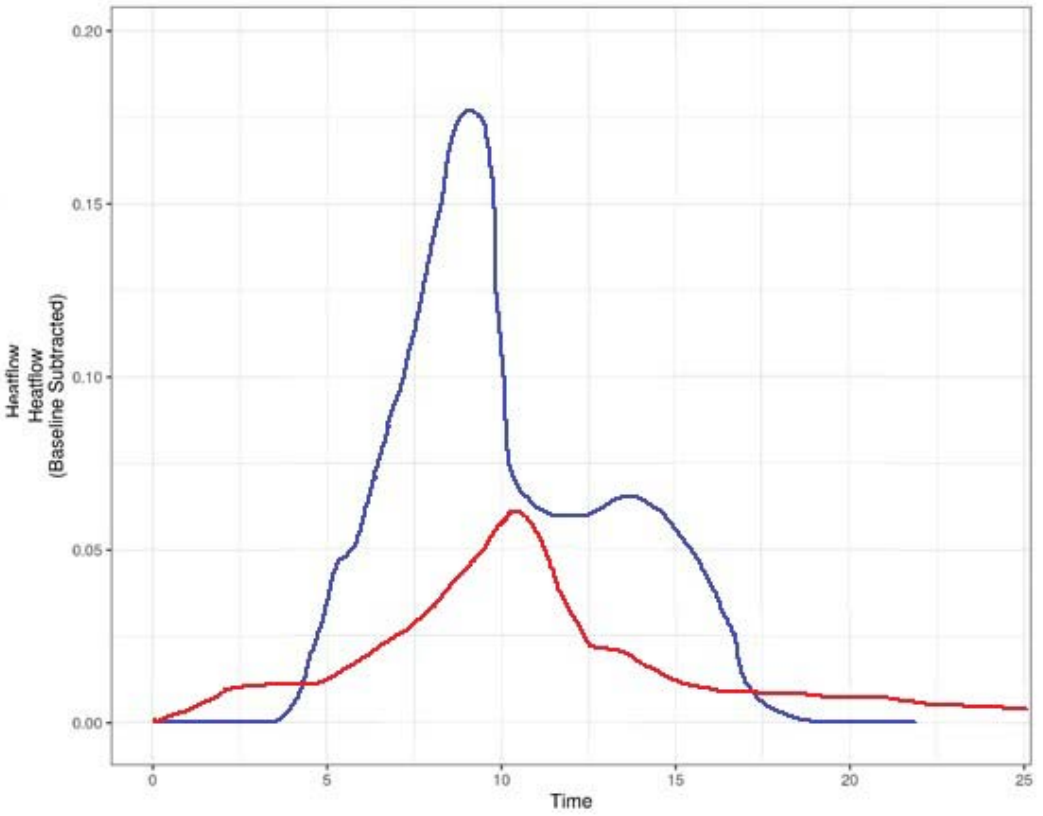

Figure 3. Graphical representation of the growth curves of the organisms studied and presented in figures 1 and 2, grouped in the same graph - to facilitate the observation of differences in thermal "footprint".

DISCUSSIONS

The physical principle underlying the operation of the calorimeter is explained by the transfer of heat between two bodies with different temperatures, called heatflow, until the two reach a thermal equilibrium. The microcalorimeter has the ability to record this calorific value by transforming it into an electrical signal, generating graphs in the form of growth curves when the heat difference is recorded between a reference and a sample[31–33].

The microcalorimeter has vats into which cells used for studies are inserted; these vats manage to isolate the cells from the external environment and provide a 3 dimensional heat sensor. The ability of the microcalorimeter to thermic isolate the cells is not perfect, for this reason it is important to maintain a constant temperature in the room where the experiments take place, because otherwise the moments of temperature difference will parasitize the signal recorded by the device. Sensitivity is beneficial for performing precise experiments, but if the working protocol is not followed, procedural flaws may occur that invalidate the experiments[32–34].

An important factor in conducting a successful experiment is related to how the experiments are designed. Formulation of medium and long-term goals is essential, because a successful experiment is one in which only one property is modified (temperature, environment, volume, bacterial dilution), and if those who intend to perform experiments do not follow a protocol well established there is the possibility of conducting experiments that do not provide valid information because they cannot be interpreted in context[10,35].

CONCLUSIONS

Microcalorimetry is a method that if applied following a very well established protocol can provide interesting information on bacterial metabolism. The applicability of this method in the medical field is possible in many areas so that the study is an important step in improving the quality of the medical act and in the diagnosis of infections.

In order to be able to use this method in the clinical study, numerous experiments are needed to be performed, following the impact of as many parameters as possible, in order to try to simulate the clinical conditions.

Once sufficient stackable experiments are cumulated, by corroborating them, a working algorithm can be created with the ability to provide information with high sensitivity, quickly and can be viewed in real time.

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