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A method for microbial decontamination of *Acanthamoeba* cultures using the peritoneal cavity of mice

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ABSTRACT

Objective: To evaluate whether the inoculation of contaminated cultures in the peritoneal cavity of mice could implement decontamination of *Acanthamoeba* cultures.
Methods: Suspensions of *Acanthamoeba*, *Acanthamoeba polyphaga* ATCC 30461, or *Acanthamoeba* spp. isolated from soil (UnB13 strain) were inoculated in the peritoneal cavity of Swiss mice ($n = 24$). After 1, 6, 12, or 24 h of exposure the peritoneal cavity was washed and assessed for the presence of bacteria, fungi, and *Acanthamoeba*.
Results: After 1 h of intraperitoneal inoculation at least 97% of the bacteria and 96% of the fungi ($P < 0.05$) and 99% of the bacteria ($P < 0.05$) were successfully eliminated from the ATCC 30461 strain and from the soil isolate UnB13 strain, respectively. This method also allowed the recovery of most trophozoites and cysts from both *Acanthamoeba* cultures at the end of 24 h.
Conclusions: Our data demonstrated that this technique has great potential for decontamination of *Acanthamoeba* cultures in a short period of time.

1. Introduction

The determination of the pathogenic potential of free-living amoebae is crucial to establish mechanisms of prevention and even to propose new therapeutic strategies for the diseases caused by these pathogens [1]. Although the isolation and cultivation of free-living amoebae collected from the environment is relatively easy, the frequent presence of contaminant opportunistic microorganisms, such as bacteria and fungi, poses an everlasting problem in the early moments of the primary isolation assays of field samples. In such circumstances, successful *in vitro* cultivation is time-consuming and requires a great deal of work. As a result, studies focusing on the pathogenicity of new isolates fail very often.

The peritoneal cavity concentrates a large population of macrophages [2,3], which are the main cells involved in the innate immune defense against microorganisms [4], including amoebae. *In vitro* assays have proven that macrophages are able to lyse or phagocytize amoebae [5], but despite the microbicidal mechanisms of macrophages to control the infection, such as the secretion of chemokines and cytokines, the *Acanthamoeba* produces peptidases that degrade these substances, also, it could lyse or induce the macrophages to apoptosis, especially when the parasites are not sensitized [4,6].

The presence of microbes in the peritoneal cavity leads to the chemotaxis of monocytes/macrophages and neutrophils, and also stimulates phagocytosis by resident macrophages and the arrival of new phagocytes to control infections. Considering that amoebae may escape from phagocytes and survive, we hypothesized that the inoculation of a culture of *Acanthamoeba* contaminated with bacteria and/or fungi in the peritoneal cavity could be an easy way for decontamination of this culture.

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Therefore, this study aimed to evaluate whether the peritoneal cavity of mice would be effective for bacterial and fungal decontamination of *Acanthamoeba* cultures.

2. Materials and methods

2.1. Acanthamoeba cultures

A culture of the reference *Acanthamoeba polyphaga* (*A. polyphaga*) strain ATCC 30461, contaminated with bacteria and fungi by manipulation, and a culture of *Acanthamoeba* spp. isolated from soil samples at the University of Brasília, UnB13 strain (GenBank database JQ268234) [1], contaminated with bacteria, were used. Both cultures were maintained at 25 °C in non-nutrient agar (Merse, São Paulo, SP, Brazil) covered with autoclaved *Escherichia coli*, without antibiotics or antifungal agents. Amoebae cysts, bacteria, and fungi were quantified in a hemocytometer.

2.2. Fungal and bacterial identification

The identification of fungal species is regularly carried out by using microscopic observation of their morphological features, especially the reproductive structures, stained with lactophenol cotton blue [7]. The fungal cells were grown in Sabouraud dextrose agar (BD-Difco, Franklin Lakes, NJ, USA), the cells were collected, stained with lactophenol cotton blue, and observed under a 40× objective of an optical microscope (Nikon Instruments Inc., Japan).

The bacterial cells contaminating the cultures of *A. polyphaga* were grown in lysogeny broth medium (1.0% tryptone, 0.5% yeast extract, 1.0% sodium chloride, pH 7.0), Gram stained, and observed under a 100× objective of an optical microscope (Nikon Instruments Inc., Japan). Several different species of Gram-positive and Gram-negative cocci and bacilli were observed. No attempts were made to identify the bacteria at species level, since they were contaminants and not the main focus of our research.

2.3. Decontamination test

The Animal Research Ethical Committee of the University of Brasilia approved the experimental protocol of this work (process number 43036/2010). Two groups of female adult Swiss mice (n = 48), aged (3.6 ± 0.5) months and weighing (27.8 ± 1.3) g, were used to evaluate the capacity of the peritoneal cavity for microbe decontamination of *Acanthamoeba* cultures. Group 1 consisted of 24 mice inoculated intraperitoneally with a contaminated culture of *A. polyphaga* ATCC 30461 strain. Group 2 consisted of 24 mice inoculated intraperitoneally with a contaminated culture of *Acanthamoeba* spp. (UnB13). The peritoneal cavity of 6 animals per time (1, 6, 12, or 24 h after inoculation) in each group was washed to assess amoeba capacity to survive and the level of decontamination of the suspension recovered. The animals were kept in a 12 h dark/light cycle, at ambient temperature, and received balanced nutrition and water, which were offered *ad libitum*.

The peritoneal cavity of Swiss mice (n = 6) was inoculated with 1 mL aliquots of suspensions of *A. polyphaga* ATCC 30461 (contaminated with bacteria and fungi) or *Acanthamoeba* spp. UnB13 strain (contaminated with bacteria) cultures, the former containing 11×10^4 amoeba cysts and the contaminants *Aspergillus nidulans* (*A. nidulans*) (28×10^5), Gram-positive and Gram-negative bacteria (13×10^6), and the latter containing 16×10^4 amoeba cysts and trophozoites, Gram-positive and Gram-negative bacteria (24×10^6) as contaminants. After 1, 6, 12, or 24 h of inoculation, six mice/group were anesthetized with 80 mg/kg of ketamine (Holliday Scott SA, Buenos Aires, Argentina) and 10 mg/kg of xylazine (Syntec do Brasil Ltda, Cotia, SP, Brazil), and the peritoneal cavity was washed with 12 mL of cold phosphate buffered saline pH 7.2. The suspension recovered from the peritoneal cavity (10 mL) of each animal was centrifuged at 1 198 r/min at 4 °C (Sorvall Mach 1.6, Thermo Scientific Sorvall, Watham, MA, USA) for 15 min and suspended in 1 mL. After homogenizing the suspension, 40 μL aliquots were analyzed in a hemocytometer chamber without previous staining to quantify amoebae, bacteria, and fungi. The results were expressed as the percentage of microbes recovered from the peritoneal cavity of mice in relation to the number of inoculated microbes.

2.4. Statistical analysis

The Bartlett's test and the Kolmogorov–Smirnov test were respectively applied to verify the homogeneity of variances and the normality of their distribution before the comparative analysis. The analyses were performed using the Kruskal–Wallis test, followed by Dunn's method to compare multiple unrelated non-normal samples. Differences with a two-tail P-value < 0.05 were considered statistically significant. The Prism 5® software package (GraphPad, San Diego, CA, USA) was employed to perform the statistical tests and the graphical design of the data.

3. Results

3.1. Recovery of A. polyphaga

The peritoneal cavity of mice was effective for bacterial and fungal decontamination of *A. polyphaga* cultures, whereas *A. polyphaga* remained alive. The percentages of amoeba cysts and trophozoites recovered from the peritoneal cavity of mice were reduced by 36.4% and 18.2% 6 h and 12 h after inoculation, respectively ($P < 0.05$), while 1 h and 24 h after inoculation the percentages recovered were similar to the inoculum (50.0% and 72.7%, respectively; $P > 0.05$) (Figure 1A).

Samples were stained with lactophenol cotton blue which were able to visualize septated and hyaline hyphae and brown conidiophores with columnar conidial heads compatible with the features described for *A. nidulans*. The percentage of fungi recovered from the peritoneal suspension, compared to the inoculum (100%), showed that regardless of the time of inoculation in the peritoneal cavity (1, 6, 12, or 24 h) the technique was efficient to remove these microbes, since the median values for all times analyzed was zero. The median of the percentage of fungi was zero in 92% of the peritoneal suspensions analyzed and, except for 2 outliers observed 1 h (0.4%) and 6 h (4.3%) after inoculation, in all the other suspensions no fungi were recovered (Figure 1B).

Our results also showed that, compared to the inoculum (100%), the percentages of bacteria present in the peritoneal suspension recovered were reduced after 1 h (0.8%), 6 h (0.0%), and 12 h (0.7%) ($P < 0.05$), but not after 24 h (2.4%) ($P > 0.05$) (Figure 1C). The elimination of 100% of bacteria was observed

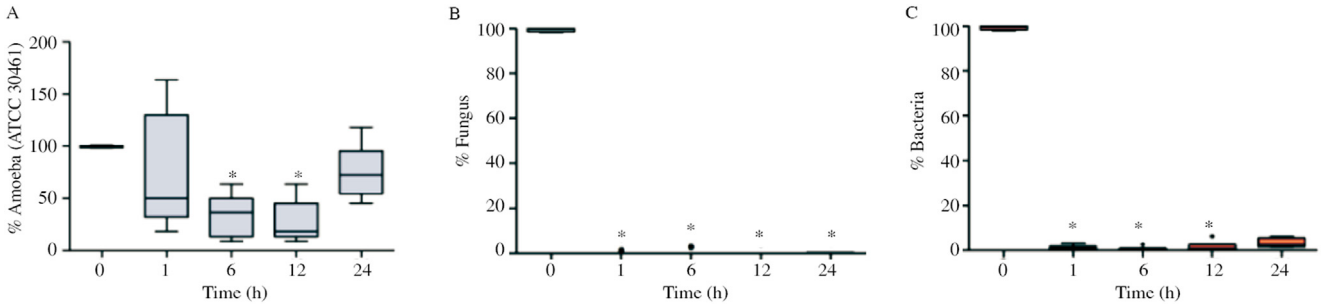

Figure 1. Quantification (%) of microbes in peritoneal suspension as a function of time.
Cultures containing (A): *A. polyphaga* (ATCC 30461) (11×10^4); (B): *A. nidulans* (28×10^5); (C): Gram-positive and Gram-negative bacteria (13×10^6) were inoculated in the peritoneal cavity of 6 Swiss mice per time of recovery ($n = 24$); The peritoneal cavity was washed and 10 mL of each suspension was recovered from the animals 1, 6, 12, or 24 h after inoculation. The results, analyzed by the Kruskal–Wallis test followed by Dunn's method, showed that the percentages of amoebae were reduced 6 and 12 h after inoculation ($P < 0.05$), the percentages of fungi were zero ($P < 0.05$), except for two outliers observed 1 and 6 h after inoculation, and the percentages of bacteria were reduced after 1, 6, and 24 h ($P < 0.05$). Data are represented as medians, quartiles, and extreme values; *: $P < 0.05$.

in 6 out of 24 peritoneal suspensions recovered, whereas in the other suspensions the elimination reached 94%.

3.2. Recovery of Acanthamoeba spp. (UnB13)

The percentages of cysts and trophozoites of *Acanthamoeba* spp. (UnB13) obtained from the peritoneal cavity after 1 h (93.8%), 6 h (109.4%), 12 h (115.7%), or 24 h (50.1%) were similar to the inoculum (100%; $P > 0.05$) (Figure 2A).

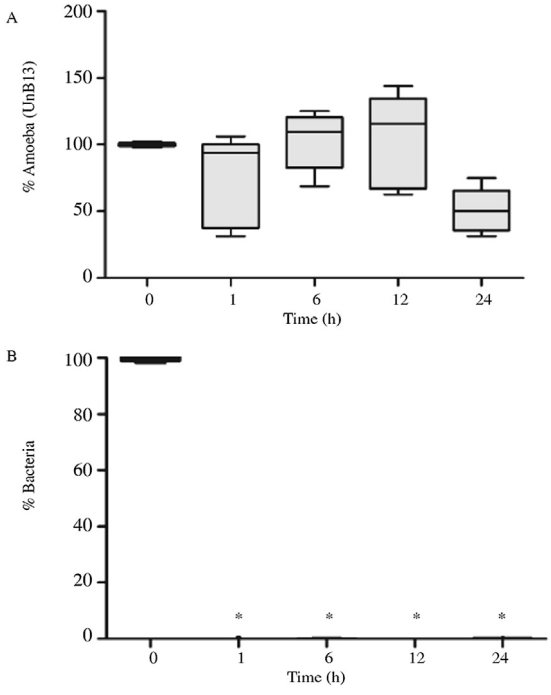

Figure 2. Quantification (%) of microbes in peritoneal suspension as a function of time.
Cultures containing (A): *Acanthamoeba* spp. (UnB13) (16×10^4); (B): Gram-positive and Gram-negative bacteria (24×10^6) inoculated in the peritoneal cavity of 6 Swiss mice per time of recovery ($n = 24$); The peritoneal cavity was washed and 10 mL of each suspension was recovered from the animals 1, 6, 12, or 24 h after inoculation to analyze the decontamination. The results, analyzed by the Kruskal–Wallis test followed by Dunn's method, showed that the percentages of recovered amoebae 1, 6, 12, or 24 h after inoculation were statistically similar to the inoculum, whereas the percentages of recovered bacteria were significantly reduced 1, 6, 12, or 24 h after inoculation ($P < 0.05$). Data are represented as medians, quartiles, and extreme values; *: $P < 0.05$.

Moreover, compared to the inoculum (100%), the percentages of bacteria present in the peritoneal suspension recovered were reduced after 1 h (0.0%), 6 h (0.2%), 12 h (0.0%), or 24 h (0.0%; $P < 0.05$) (Figure 2B). In 16 out of 24 peritoneal suspensions analyzed, 100% of the bacteria initially present were eliminated, while in the remaining 8 peritoneal suspensions recovered the elimination reached 99.8%.

4. Discussion

The present study showed, for the first time, the potential utility of the peritoneal cavity of mice for decontamination of *Acanthamoeba* cultures. According to our data, 1 h after intraperitoneal inoculation, at least 97% of the bacteria and 96% of the fungi and 99% of the bacteria were successfully eliminated from the ATCC 30461 strain and from the soil isolate UnB13, respectively. All the fungi were eliminated in 92% of the peritoneal suspensions recovered from the animals inoculated with the ATCC 30461 strain. All the bacteria were eliminated in 67% of the peritoneal suspensions recovered from the animals inoculated with the UnB13 isolate. Although the peritoneal cavity was effective for decontamination of both samples of *Acanthamoeba*, our results showed a decrease in the percentage of recovery of the strain ATCC 30461 6 or 12 h after intraperitoneal inoculation, while the recovery of the soil isolate UnB13 did not differ from the inoculum at the different times tested.

Conventional antibiotics normally eliminate contaminating microbes, but their persistence in some cultures can cause difficulty in studies involving amoebae, since these microbes may acquire resistance to these antibiotics, and therefore the contaminated cultures are commonly discarded in laboratories of research, leading to sample loss. Given that this study proved intraperitoneal inoculation to be a fast and efficient method for decontamination of amoeba cultures, and considered the difficulties encountered for isolating amoebae from contaminated cultures with microorganisms that are resistant to conventional antibiotics, this technique may be of great importance for the continuity of such studies.

The efficiency of peritoneal macrophages to recognize and eliminate microbes was observed in the peritoneal suspensions recovered from most animals inoculated with *Acanthamoeba* (ATCC 30461 and UnB13) cultures contaminated with bacteria and/or fungi. The peritoneal cavity is rich in macrophages [2], and the innate immune defense is triggered when the host is in contact with infectious agents, such as bacteria and fungi,

activating these cells. Macrophages express both receptors that recognize ligands (molecular patterns) on the cell membranes of microbes and receptors that bind to opsonins, such as complement components and the Fc portion of immunoglobulin G. This recognition promotes the process of endocytosis of microbes by forming pseudopodia and, once ingested, they are subjected to the enzymatic complex of lysosomes [8,9] and to reactive oxygen and nitrogen toxic molecules [10–13]. Concomitantly, macrophages recruit other leukocytes to the site of infection [14] by releasing cytokines and chemokines. It is possible that the coordinated action of lysosomal enzymes, production of reactive oxygen and nitrogen species, cytokines and chemokines contributed for bacterial and fungal decontamination of the suspensions recovered from the peritoneal cavity of mice.

Although the method employed in this study eliminated most bacteria, 2.4% of the contaminant bacteria still persisted in the suspension obtained 24 h after inoculation with *A. polyphaga* ATCC 30461, while at least 99.8% of the bacteria contaminating the *Acanthamoeba* spp. (UnB13) culture were eliminated. The higher percentage of bacteria that remained in the peritoneal cavity of animals inoculated with ATCC 30461 culture evidences the possible higher virulence of microbes that have been selected for a long time in culture media with antibiotics. On the contrary, the bacteria found in the soil isolate UnB13 were more easily subjected to the microbicidal mechanisms of macrophages, possibly due to their lower virulence. Another possibility to explain the increased virulence of bacteria is their endosymbiotic interaction with *Acanthamoeba* [15].

It is important to note that, similarly to the bacteria and fungi, both *Acanthamoeba* samples were also exposed to the same microbicidal mechanisms of the phagocytes during the time they remained in the peritoneal cavity. However, our results showed that they escaped from these defense mechanisms and most of them were recovered up to 24 h after inoculation. This result may demonstrate the resistance of *Acanthamoeba* cysts to alterations in temperature, pH, and antimicrobial actions [16]. These findings suggested the efficiency of 2 layers of polysaccharides (cellulose) in the cell wall of amoeba cysts to escape from phagocytes [17,18]. Despite escaping from recognition and elimination by phagocytes, it is possible that part of the inoculated cysts were eliminated by phagocytic cells.

The similar percentages of *Acanthamoeba* spp. (UnB13) collected from the peritoneal cavity 6 h (103.2 ± 21.9)% or 12 h (106.3 ± 33.5)% after inoculation, compare to the inoculum, could result from the reproduction of trophozoites or their transformation from the cysts in the peritoneal cavity of mice. This suggested that trophozoites could multiply into the peritoneal cavity even when subjected to phagocytosis by peritoneal macrophages. However, despite the presence of trophozoites in the inoculum of *Acanthamoeba* spp. (UnB13), our results showed that 24 h after inoculation their number was reduced almost by 50% in the peritoneal suspension. This result demonstrated the possible low resistance of trophozoites to the microbicidal mechanisms of phagocytes, since they have a single layer of polysaccharide in their cell membrane [19]. Another plausible explanation would be that part of the inoculated cysts left the peritoneal cavity and reached other organs via bloodstream.

In conclusion, our data demonstrated that the procedure using the peritoneal cavity of mice has potential for bacterial and fungal decontamination of *Acanthamoeba* cultures. Besides eliminating bacteria and fungi, this method allows the recovery of cysts in a short period of time and promotes the acquisition of trophozoite forms in the peritoneal suspension. Our results point to an up-and-coming technique to be employed in parasitological research at low cost, with high efficiency, and accessible even in modest laboratories.

Conflict of interest statement

We declare that we have no conflict of interest.

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