The Effect of Cell-Free Supernatants of Free-Living Amoeba against Some *Staphylococcus* Bacteria: First Findings from Turkey

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ABSTRACT

**Objective:** Free-living amoeba (FLA) are protozoa living in soil and in natural and man-made water systems. They attract much attention owing to the illnesses associated with them and to their relationships with bacteria. In this study, the effect of cell-free supernatant (CFS) obtained from FLA against *Staphylococcus* was investigated.

**Materials and Methods:** Environmental FLA strains (A1, A2, A3) were obtained from lake water and swimming pools in Istanbul. *Acanthamoeba castellanii* ATCC 50373 was used as the standard strain. Clinical *Staphylococcus* strains (S1, S2, S3) were obtained from a culture collection at Istanbul University, Faculty of Pharmacy, Department of Pharmaceutical Microbiology. As standard strains, MRSA ATCC 43300, *S. epidermidis* ATCC 12228, *S. aureus* ATCC 29213 were used. FLA-CFS were obtained by centrifuging and filtering of axenic cultures. Colony counting technique was used to investigate the inhibition activities of FLA-CFS against *Staphylococcus* bacteria.

**Results:** Against MRSA ATCC 43300 strain, CFSs of *A. castellanii* ATCC 50373 and A1 showed an inhibition efficiency of 78.36% and 73.47%, respectively. Against S1 strain, CFSs of *A. castellanii* ATCC 50373 and A2 showed an inhibition of 65.64% and 15.14%, respectively. Against *S. aureus* ATCC 29213, only A1-CFS showed inhibitory effect (44%). It was found that *A. castellanii* ATCC 50373 and A2-CFSs inhibited the S2 strain 26.20% and 9.24% respectively. Against S3 strain, A2-CFS was inhibitory at 33.33%. No FLA-CFS could be inhibitory against *S. epidermidis* ATCC 12228.

**Conclusion:** It is necessary to devise new studies in which sample numbers are increased when using FLA strains in the inhibition of antibiotic-resistant bacteria.

**Keywords:** Free-living amoeba, *Staphylococcus* infections, *Acanthamoeba*, antibacterial effect, cell-free supernatant, antibiotic resistance

INTRODUCTION

Free-living amoeba (FLA) are eukaryotic microorganisms living in soil, air, sea water, fresh water and man-made water systems (pools, cooling towers, water pipes, etc.) (1-8). They have two stages in their lifecycle, namely trophozoite and cyst (9). Trophozoites are metabolically active, feeding, mobile forms which transform into cysts. The latter are inactive metabolically and exist in adverse environmental conditions (starvation, temperature changes, pH changes, etc.) (10). Pathogenic FLA for humans are *Naegleria fowleri*, *Acanthamoeba spp.*, *Balumuthia mandrillaris*, and *Sappinia spp.* (5, 11). Along with their pathogenicities, FLA also attracted scientists’ attention due to their interactions with other microorganisms in the environments in which they live. In this collective living, they play important roles in the
regulation of microorganism population and microbial ecology (12, 13): i) They use some bacteria, fungi, and algae as food (10, 14, 15). ii) Some bacteria which can enter inside FLA find themselves isolated from adverse environmental conditions (antibiotics, disinfectants, etc.) and they find the opportunity to multiply (16-19). After this, they can cause lysis of FLA cells (20). iii) They can inhibit some bacteria which does not enter inside FLA cells (non-fagocyting). Very scarce studies in recent years show these abilities (21-24).

Recently, increasing antibiotic resistance has become an important problem in the treatment of bacterial infections. Every year, about 2.9 million infections owing to the presence of antibiotic-resistant bacteria occur and about 36 thousand of them end in death (25). From past to present, antibacterial substances have been isolated from organisms like bacteria, fungi, algae, insects, and plants (26-29).

As is the case all around the world, new antibacterial compounds are also sought after in our country because antibiotic-resistant bacteria cause important problems in the treatment of these infections. However, the number of studies showing the antibacterial effect of FLA is quite limited in the literature (22, 23). In this context, this research aims to look for the antibacterial effect of cell-free supernatant (CFS) of FLA strains isolated in Turkish waters against infection-agent Staphylococcus bacteria.

**MATERIALS AND METHODS**

**Test Microorganisms**

In this study, the antibacterial activity of CFS obtained from four FLA (Acanthamoeba castellanii ATCC 50373, A1, A2, A3) was investigated against six Staphylococcus strains [Methicillin-resistant Staphylococcus aureus ATCC 43300 (MRSA ATCC 43300), Staphylococcus aureus ATCC 29213 (S. aureus ATCC 29213), Staphylococcus epidermidis ATCC 12228 (S. epidermidis ATCC 12228), Methicillin-resistant Staphylococcus aureus S1 (S1), Methicillin-sensitive Staphylococcus aureus S2 (S2), Methicillin-resistant Staphylococcus epidermidis S3 (S3)] (Table 1).

Among the FLA strains used in this study, two of them (A1 and A2) were isolated from swimming pools in a previous study of ours (8), one of them (A3) was isolated from lake water, and A. castellanii ATCC 50373 was used as standard strain. Clinical Staphylococcus strains (S1, S2, S3) used in our study were obtained from the culture collection at Istanbul University (I.U.) Faculty of Pharmacy, Department of Pharmaceutical Microbiology. MRSA ATCC 43300, S. aureus ATCC 29213 and S. epidermidis ATCC 12228 were used as the standard strains.

**Bacterial Cultures**

MRSA ATCC 43300, S. aureus ATCC 29213, S. epidermidis ATCC 12228, S1, S2 and S3 strains were cultured in Trypticase soy agar (TSA) at 37°C for 24 hours and used in the experiment.

**FLA Cultures**

FLA strains (A1, A2) previously isolated and kept in the freezing medium (FM) at -86°C were first brought to room temperature. Petri dishes containing non-nutrient agar (NNA) spread on Escherichia coli (E. coli) pre-inactivated (121°C, 15 minutes) were used for the resuscitation of the strains. All Petri dishes were incubated at 30°C.

**Table 1. Microorganisms used in antibacterial activity experiments.**

| Microorganisms (Code) | Source |
|-----------------------|--------|
| **FLA Strains** | | 
| Acanthamoeba castellanii ATCC 50373 | Standard Strain |
| A1 | Swimming pool isolate |
| A2 | Swimming pool isolate |
| A3 | Lake water isolate |
| **Staphylococcus Strains** | | 
| MRSA ATCC 43300 | Standard Strain |
| S. aureus ATCC 29213 | Standard Strain |
| S. epidermidis ATCC 12228 | Standard Strain |
| S1 | Clinical isolate |
| S2 | Clinical isolate |
| S3 | Clinical isolate |

*a: Methicillin-resistant Staphylococcus aureus ATCC 43300, b: Methicillin-sensitive Staphylococcus aureus ATCC 29213, c: Methicillin-sensitive Staphylococcus epidermidis ATCC 12228, d: Methicillin-resistant Staphylococcus aureus, e: Methicillin-sensitive Staphylococcus aureus, f: Methicillin-resistant Staphylococcus epidermidis.*
First, A3-coded amoeba strain was isolated from Uluabat Lake (Bursa, Turkey) under aseptic conditions, and the water sample in a glass bottle was concentrated by passing through a Sartorius filtering device having a membrane filter (0.22 µm). Then, the filter paper was turned upside down and left on fresh NNA medium having inactivated *E. coli* and incubated at 30°C (4, 34). Petri dishes were examined on an inverted microscope on a daily basis.

**FLA-Axenic Culture and FLA-CFS**

To obtain FLA-axenic cultures, Pepton Yeast Extract Glucose medium (PYG) containing Page’s amoeba saline (PAS) solution (PYG-PAS) was used (11, 35). Antibiotics (0.5 mg/mL penicillin and streptomycin) were added to PYG-PAS to avoid microbial contamination. Amoeba cells in the Petri dishes were collected with 2-3 mL of PAS solution and inoculated into T-25 tissue culture flask containing antibiotic-added PYG-PAS and incubated at 30°C (22, 36). When the mono-layered axenic culture was observed, the PYG-PAS containing antibiotics in the tissue culture flask was discharged and washed with fresh PAS three times, in order to clean the culture from antibiotics (17). After the last washing, fresh PYG-PAS was placed into the flask and incubated for 48-72 hours at 30°C. The FLA-axenic culture thus obtained was subjected to Thoma slide and trypan blue for counting (cell/mL) and vitality determination (37, 38). Cell-free supernatant of FLA (FLA-CFS) was obtained after centrifuging the FLA-axenic cultures (1000 g x 5 min) followed by passing the supernatant through 0.22 µm pore-diameter filters and were used in antibacterial experiments (22, 23).

**Antibacterial Activity Experiments**

Colonial counting method was used to assess the antibacterial effect of FLA-CFSs against *Staphylococcus* bacterial strains (22, 23). Bacterial suspension (10^6 cfu/mL) in phosphate buffered saline (PBS) and FLA-CFS were mixed in a tube (1:10) and incubated for 18-24 hours at 37°C. After incubation, each mixture was diluted further with PBS 10 times to achieve a series of dilutions (10^-1–10^-6) and spread into Petri dishes containing TSA. All petri dishes were incubated for 24 hours at 37°C and after the period, colonies were counted. In the experiments, PYG-PAS and PBS were used as control groups. All experiments were performed in triplicate.

**RESULTS**

**FLA-Axenic Culture and FLA-CFS**

Figure 1 shows the images obtained under inverted microscopic examination (x100) of FLA-axenic cultures.

Each FLA-axenic culture had the following living FLA trophozoite numbers: For *A. castellanii* ATCC 50373, A1, A2, A3, the values are 3x10^5, 8x10^5, 5x10^5, 1x10^5 cell/mL, respectively. Trypan blue-dyed FLA-axenic cultures had the microscopic view (x100) of undyed/living FLA trophozoites in Figure 2.

**Antibacterial Activity**

The tested FLA-CFSs showed the highest inhibition effect on the MRSA ATCC 43300 strain among the *Staphylococcus* species tested (Figure 3). *A. castellanii* ATCCC 50373 coded FLA (*A. castellanii* ATCCC 50373-CFS) and A1 encoded FLA-CFS (A1-CFS) inhibited MRSA ATCC 43300 bacteria by 78.36% and 73.47%, respectively. *A. castellanii* ATCC 50373 and A2-encoded FLA-CFS (A2-CFS) inhibited the S1 strain 65.64% and 15.14%, respectively. Against *S. aureus* ATCC 29213, only A1-CFS showed inhibitory effect (44%). It was found that *A. castellanii* ATCC 50373-CFS and
A2-CFS inhibited the S2 strain 26.20% and 9.24% respectively. Although A2-CFS inhibited S3 strain (33.33%), other FLA-CFSs did not show antibacterial effect against this strain. None of the tested FLA-CFSs were found to have an inhibitory effect on S. epidermidis ATCC 12228 strain. A3-CFS was found to have no inhibitory effect on any Staphylococcus strain tested. However, it was determined that Staphylococcus strains tested were inhibited by at least one native FLA-CFS (A1-CFS or A2-CFS), except for S. epidermidis ATCC 12228 strain (Figure 3).

According to these data, it was understood that FLA-CFS had more inhibitory effect against S. aureus than S. epidermidis generally. S. epidermidis ATCC 12228 strain was not found to be inhibited by any FLA-CFS.

**DISCUSSION**

FLA have been attracting the attention of scientists for many years due to their role of regulating microorganism populations while living together with bacteria in the same environment. In the literature, FLA-bacteria relationship has frequently centered on the abilities of amoeba phagocytizing bacteria, proliferation of bacteria in amoeba and therefore, causing lysis of them (16-19, 39, 40). However, there are few studies with narrow scope about amoeba inhibiting the proliferation of bacteria by secreting antibacterial substances (apart from phagocytosis) (21-24, 41, 42). Increasing the number of studies on this topic would help researchers to understand the FLA-bacteria relationship and would enhance microbial ecologic studies, leading to the discovery of new antibacterial substances for bacteria (especially antibiotic-resistant bacteria), which has been a great burden in these times. It was determined that the FLA tested in our study had an inhibitory effect on S. aureus and S. epidermidis (except phagocytosis). The highest inhibition against Staphylococcus bacteria (65.64%, 78.36%) was detected with A. castellanii ATCC 50373-CFS. A native aquatic FLA strain obtained from our waters (A1-CFS) also yielded a similar (73.47%, 44%) inhibition effect. On the contrary, with the other native samples (for A2-CFS) these inhibitions were either low (15.14%, 9.24%, 33.33%) or (for A3-CFS) non-existent not at all (Figure 3). Nevertheless, these data show us that native FLA have the antibacterial potential against Staphylococcus bacteria. This study is the first one about the interaction/inhibition of FLA-Staphylococcus in which native strains were used. Our studies are ongoing, aiming to investigate more samples to reveal more bacteriostatic or bactericidal effects of FLA isolates against pathogen/potentail pathogen and antibiotic-resistant Staphylococcus. If this kind of effect were found, a new, native, and effective antibacterial substance, which would be applied to inhibition of Staphylococcus that are causing infections in humans, would be discovered.

Similar to our study, Nakışah and Chandrika reported that clinical and environmental (two sample) Acanthamoeba (FLA) lysates showed antibacterial activity against pathogen two S. aureus strains (21). Iqbal and co-workers reported that A. castellanii-CFS (one sample) was effective against clinical MRSA at 100% whereas it had a bactericidal activity against vancomycin-resistant E. faecalis at 8%. However, it did not show a bactericidal effect against Acinetobacter sp., Pseudomonas aeruginosa (22). Souza et al. reported that the relationship between a single clinical MRSA isolate and a single Acanthamoeba polyphaga ATCC 30461 (FLA) strain yielded that FLA culture lysate supported the growth of MRSA, but the same FLA culture supernatant inhibited the growth of the same bacteria (24). Martin et al. investigated the effects of different genus, kind, and origin of FLA-CFS against Staphylococcus aureus (MRSA typed as USA300) and found that Mycobacterium bovis biofilms and A. polyphaga CCAP 1501/18–CFS sample inhibited Staphylococcus aureus to a significant degree (42). As can be seen, there is a very low number of current studies testing a scarce number of strains. However, many more strains (four FLA strains and six Staphylococcus strains) were used in our study, thus making it more comprehensive. Moreover, our study used strains different from the standard strains used in other studies, including native strains. For these reasons, we are of the opinion that the inhibitory effect of FLA CFSs on Staphylococcus bacteria may vary depending on both FLA strains and Staphylococcus strains. The following data obtained from our study (Figure 3) also support this view: i) The tested FLA-CFSs had a greater inhibitory effect against S. aureus than S. epidermidis. ii) No FLA-CFS had antibacterial activity against S. epidermidis ATCC 12228 strain. iii) A3-CFS had no inhibitory effect on any of the Staphylococcus strains tested. iv) Staphylococcus strains tested were found to be inhibited by at least one native FLA-CFS (A1-CFS or A2-CFS), except for the S. epidermidis ATCC 12228 strain). Nevertheless, new studies are needed in which many more Staphylococcus and FLA strains would be used. This would be the only way to shed further light on the subject.

As stated above, since it is thought that the characteristics/abilities (pathogenicity factors) possessed by these microorganisms may play an important role among the factors affecting the relationship between FLA and bacteria living in the same environ-
ment, the subject should be investigated in detail. For example, it is known that the S. epidermidis ATCC 12228 strain, which was tested in our study and is avirulent, has molecules called bacteriocin that it releases into the external environment (43). This bacterium thus inhibits some species (eg S. aureus) that live in the same environment and are close to it. In our study, the reason why S. epidermidis ATCC 12228 strain was not inhibited by any FLA-CFS is perhaps because of these and similar molecules owned by the bacterium. In addition, it was found that the clinical (virulent) S. epidermidis strain (53) tested in our study was inhibited by A2-CFS to a low extent (33.33%). These data suggest that virulent and avirulent S. epidermidis strains may be affected in different ways by FLA-CFS. In order to better understand the inhibition effect of FLA-CFS against S. epidermidis, new studies are planned in which more strains would be used (both virulent and avirulent S. epidermidis).

Souza Gonçalves et al. found that A. castellanii secreted different extracellular vesicles under different stress conditions and the CFS of these vesicles contained 69 proteins (41). In the light of this information, it is thought that the amoeba strains used in our study secrete inhibitory molecules against Staphylococcus strains, and new studies are planned to find the morphological and biochemical characterization of these possible molecules. Subsequent studies should relate to the purification of FLA-CFS with such activity and the active compound(s) that helped in the discovery of new anti-Staphylococcus active compound(s).

CONCLUSION

This is the first study showing the anti-Staphylococcus potential of FLA-CFS isolated from Turkey. In order to discover bactericidal FLAs against pathogen/potential pathogen and antibiotic resistant Staphylococcus bacteria, new studies using more strains should be planned.

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