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ANTIMICROBIAL RESISTANCE AND ENDOSYMBIOSIS IN *ACANTHAMOEBA*

TREBALL DE FI DE GRAU

Carla Donet Donet
Bioquímica i Biologia Molecular
Academic Advisor: Sandra Guaita Esteruelas
Internship Advisor: Fiona Henriquez
Carla.donet@estudiants.urv.cat
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ABSTRACT

The overproduction and uncontrolled use of antibiotics has produced one of the most important current clinical dilemmas. Increased contamination of wastewater in low-middle income countries such as India has triggered an evolutionary process in bacteria, which through horizontal gene transfer has increased antimicrobial resistance (AMR). In addition, many bacteria are resistant to phagocytic mechanisms of other microorganisms such as amoeba and initiate endosymbiotic interactions with them. Understanding these relationships is important to monitor AMR environmentally and clinically. Therefore, different antibiotics found in high concentration in the waters of Vashi Creek in India were used to observe AMR bacteria in these and the understand how the endosymbiotic effect with amoebae is influencing it. The techniques used were based on the isolation of extracellular and intracellular bacteria from amoeba for comparison of the effect of antibiotics on both, followed by determination of the Minimum Inhibitory Concentration (MIC) and comparison of results in both groups of bacteria. Sulfamethazine antibiotic obtained high resistance results for bacterial strains isolated from within the amoeba and was therefore tested in the MIC test for three study bacteria: *E. coli*, *Ps. aeruginosa* and *Ps. putida* and the amoeba: *Acanthamoeba castellanii* ATCC 50370. Higher resistance results were shown in the MIC test for amoeba-bacteria coexistence than when bacteria were tested alone. Then, the presence of the amoeba and bacterial species was tested in clinical samples from *Acanthamoeba* Keratitis patients to ensure that this hypothesis can be translated to the clinical side. Finally, it was concluded that the bacteria can inhibit the phagocytic effect of the amoebae in order to stay inside and use the amoeba as a host, which provides them with increased protection to some antibiotics.

1. INTRODUCTION

1.1. Antimicrobial resistance and contributing factors

1.1.1. Antibiotic overuse

The introduction of antimicrobials into the clinical sector became one of the most important and necessary medical interventions for the development of complex medical approaches (Munita and Arias, 2016). Indeed, it has been one of the major therapeutic agents against bacterial, fungal, parasitic, and viral infections. However, their overuse has been directly linked to the rise of antimicrobial resistance (AMR), which is presently one of the major challenges to human health. (Davies and Davies, 2010; Larsson and Flach, 2021).

Antimicrobials include antivirals, antifungal, antiparasitic and antibacterial, commonly known as antibiotics. The use of the antibiotics is determined by several factors such as clinical, biological, social, political, economic, and environmental which make it difficult to regulate. These lead to the observation of cases of misuse and overuse, self-medication or even use of antibiotics in animals (Kivumbi and Standley, 2021). Furthermore, if the biological factor is noted, the lack of information on genetic and biochemical processes in the early stages of an epidemic bacterial infection has led to the increased horizontal transfer of a wide range of antibiotic resistance genes (**figure 1**) (Davies and Davies, 2010). This results in pathogens recurrently acquiring new resistance factors from other species, thereby reducing the ability to prevent and treat infections (Larsson and Flach, 2021).

It has also been observed that the uncontrolled increase in the production of natural, semi-synthetic or synthetic antimicrobial compounds approved so far have resistance to at least one of the pathogens targeted. This suggests that external factors already present a level of resistance to antibiotics, thus increasing the pathogenicity of the microorganisms (Larsson and Flach, 2021).

1.1.2. Heavy metals

While the emergence of AMR is largely associated with misuse or overuse of antibiotics, it is not the only factor. It is widely accepted that the chemical industry may contribute to AMR in the environment through the discharge of waste into waterways, including rivers, streams, and oceans. This has resulted in a gradual increase in the concentration of heavy metals such as copper, zinc, lead, or cadmium (**figure 1**) in the water, which are highly toxic to humans and bacteria. However, it has been observed that the microorganisms present in these waters have learned to tolerate them by showing a kind of metal resistance which has been highly compared to the antibiotic mechanism (Eggers, Safdar and Malecki, 2018; Hubeny *et al.*,2021).

In addition, one of the main problems of these heavy metals is their long degradation time, which can accumulate and exert a long-term selective pressure. It has been observed that, in the presence of heavy metals, they may be able, through horizontal gene transfer, to induce co-selection mechanisms to activate the expression of more than one antibiotic resistance gene leading to microorganisms with multi-drug resistance and thus increase colonisation and infection of the associated microorganisms (Eggers, Safdar and Malecki, 2018; Hubeny *et al.*,2021).

1.1.3. Interactions between microorganisms

It is also rarely considered when discussing AMR that natural spaces such as water, air or soil are composed of a great variety of microorganisms like bacteria, amoebae, algae, or fungi which interact in complex ways. The natural environment produces interactions between the different species (**figure 1**) that allow them to survive or feed on each other (Okude *et al.*, 2012). Such interactions allow the genetic factors that determine AMR to be passed from one species to another. Besides, it is hypothesised that mutualistic or endosymbiotic relationships between species may share these genes and help and protect each other to produce increased resistance (Manca, Farias and Aredes Fernández, 2020).

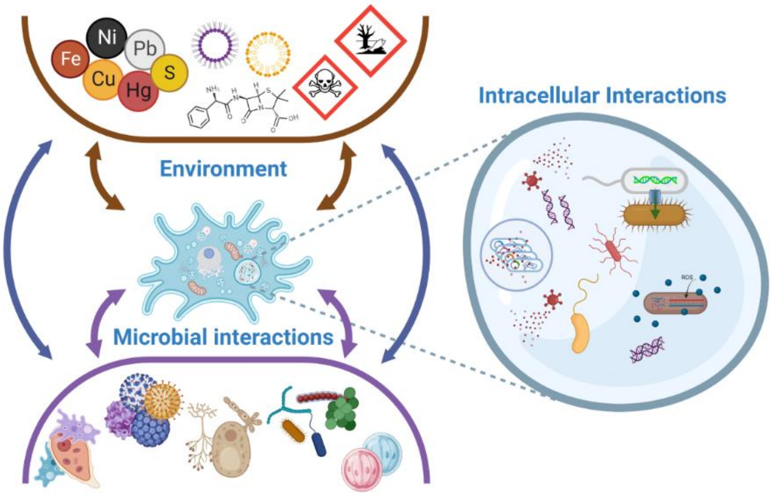


Figure 1. Interaction of bacteria in the environment. The image describes the interactions that bacteria experience with contaminated environments, with other types of microorganisms, and intracellular and evolutionary changes that it can develop. (Created by Biorender, Dr. Stuart Woods, UWS)

1.2. India and current efforts to combat antimicrobial resistance

1.2.1. How antimicrobial resistance is affecting India?

India is low to middle-income country (LMIC) located in South Asia which has a high demography, and the economy is mainly based on industry with many textile and chemical factories. There are many challenges due to industry development, including short-term pollution which can induce various health risks and environmental pollution of water and soils. In addition, the country has one of the highest rainfall rates in the world during the monsoon season (June to October) where high levels of pollution run off into the water (*India | National Geographic,* no date; Dutta and Jinsart, 2022). This makes India one of the most polluted countries in the world in terms of air, soil, and water pollution, leading to dangerous AMR related human health problems resulting in more than 50,000 new-born deaths per year (Dixit *et al.*, 2019).

1.2.2. Policies in place and strategies to combat antimicrobial resistance

The increase of contamination in these underdeveloped countries and the direct relationship with the increase of AMR is presented as a clinical threat which is intended to be combated through measures or strategies to reduce it. For this end, a series of antimicrobial stewardship and surveillance programmes has been devised, as well as susceptibility tests for resistance genes to study how different microorganisms increase their resistance and what new treatments could be used to combat them. However, many of the AMR factors are challenging to address in LMIC. Therefore, one of the main focuses is on health policy in these countries by avoiding informal and over-the-counter use of drugs as well as financial support and studies in rural areas to achieve global results and reduce many infectious diseases (Kivumbi and Standley, 2021).

1.3. Amoeba and antimicrobial resistance

1.3.1. *Acanthamoeba* species

Acanthamoeba species are free-living protists of the Amoebozoa phylum that are widely distributed in the air, soils, freshwaters, and highly contaminated habitats (Okubo *et al.*, 2018). There are more than thirty described species in the genus *Acanthamoeba* and are currently classified in 20 genotypes (T1-T20) (Behera, Satpathy and Tripathi, 2016; Jercic *et al.*, 2019)

There are two morphological stages of *Acanthamoeba* depending on the life cycle: the trophozoite and the cyst (**figure 2**). The trophozoite is the active and reproductive stage of the amoeba. It is approximately 15-35 µm in size and is characterized by the presence of pseudopods that allow movement and facilitate the phagocytosis procedure. In addition, it has a central nucleus and a contractile vacuole which is in the cytoplasm with the functionality of controlling the water content inside the cell. The reproduction mechanism of these microorganisms is done by binary fission. (Marciano-Cabral and Cabral, 2003; Roberts and Henriquez, 2010; Henriquez *et al.*, 2021)

However, when the amoeba is in adverse environmental conditions such as food deprivation, desiccation and changes in the temperature and pH, it starts the cell differentiation process of encystment from trophozoite to cyst. (Marciano-Cabral and Cabral, 2003; Samba-Louaka, 2023). The cyst state is a dormant state of the amoeba that is caused to reduce metabolic activity for survival (McBride *et al.*, 2005). Morphologically, it is approximately 10-20 µm in size and is characterized by the formation of a double cellulose cell wall, an irregular, thick outer cell wall (ectocyst) and a thin, smooth inner cell wall (endocyst) that provides increased protection from environmental stressors (Pérez-Irezábal et al., no date; Henriquez *et al.*, 2021).

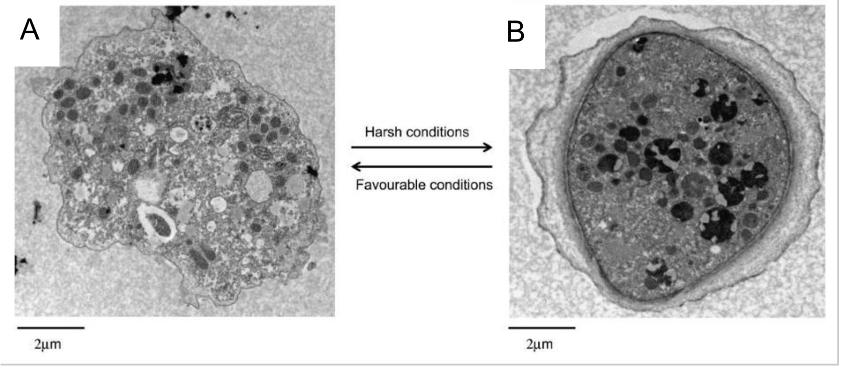


Figure 2. Trophozoite and cyst states of the *Acanthamoeba*. The image A represents the trophozoite state and the image B represents the cyst state. (Siddiqui, 2012).

1.3.2. Relevance of *Acanthamoeba* clinically

Acanthamoeba keratitis (AK) is a disease caused primarily by genotypic T4 *Acanthamoeba* species (Castro-Artavia *et al.*, 2017). Some of the most pathogenic species are: *A. castellanii* (T4) *A. polyphaga*,(T2,T4) and *A. rhysodes*,(T4), but it can also be affected by *A. hatchetti*, (T11) *A. culbertsoni*, (T10) and *A. griffini*, (T3) (Marciano-Cabral and Cabral, 2003; Ledee *et al.*, 2009; Putaporntip *et al.*, 2021). This is a progressive disease, attributed principally to the misuse of contact lenses and it’s manifested as painful sight-threatening infection of the cornea than can lead to permanent visual impairment or blindness (McBride *et al.*, 2005; Lorenzo-Morales, Khan and Walochnik, 2015; Neelam and Niederkorn, 2017). The first case of AK was reported in 1974 and since then, the number of people infected has been increasing with an actual annual incidence of 23,561 cases equivalent to 2.9 cases per million where India has the highest incidence with a total of 15.2 cases per million people. (Zhang *et al.*, 2023)

The **pathogenic mechanism** of *Acanthamoeba* is one of the main targets of study, as a complete understanding of this process could lead to new diagnostics and thus to effective therapeutic alternatives (Lorenzo-Morales, Khan and Walochnik, 2015). The infection process begins when the amoeba*,* mainly coming from contact lenses, makes first contact with the host's cornea, and gets stuck in it. It's seen that contact lens fluid with poorly regulated exchange or cleaning has high concentrations of filth, creating an ideal environment for the pathogen (Lorenzo-Morales, Khan and Walochnik, 2015). Besides, contact lens wear alters the ocular surface, making it more receptive to trophozoite attachment and increases the expression of hand-mediated glycoproteins on the surface of the corneal epithelium (Neelam and Niederkorn, 2017)

This adhesion between the amoeba and the corneal epithelium is done to cross the biological barrier of the host. For this purpose, different adhesins, mannose-binding proteins and laminin-binding proteins have been identified in the process, which bind to specific receptors on the host cell. Toll-like receptor Toll-4 (TLR4) that has been shown to be one of the receptors that provide specific binding to *Acanthamoeba* (Lorenzo-Morales, Khan and Walochnik, 2015; Neelam and Niederkorn, 2017). The specific binding sites in the cornea have led to the concept of *Acanthamoeba*-host specificity (Neelam and Niederkorn, 2017). Then, the amoeba initiates the process of phagocytosis, (**figure 3**) a mechanism of absorption of food particles necessary for its survival and reproduction. In this procedure, the pathogen affects the host signalling pathways leading mainly to cell cycle sensing and inhibition of related genes resulting in apoptosis of host epithelial cells (Neelam and Niederkorn, 2017).

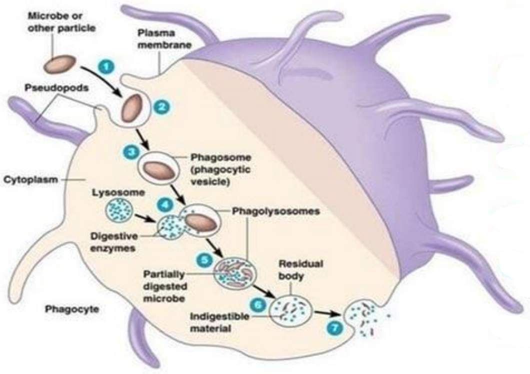


Figure 3. Process of phagocytosis. Steps: (1) Detection of a microbe or other particle. (2) Engulfment of the microbe through the plasma membrane. (3) Phagosome formation (phagocytic vesicle). (4) Phagolysosome formation (fusion with lysosome). (5) Digestion of microbe in the phagolysosome. (6) End of the digestion, formation of residual body. (7) Discharge of waste material. (Aryal, 2022).

In advanced stages of infection, the amoeba proceeds by the mechanisms of phagocytosis and binary fission, spreading throughout the epithelial and stromal zone of the cornea to cause severe inflammation in the host. In this state, some of the prominent symptoms of AK disease are redness of the eye, pain with photophobia, lacrimation, ring-shaped stromal infiltrate composed of infiltrating inflammatory cells, epithelial defect, and eyelid oedema. In extreme cases of infection, a corneal ulceration may be developed leading to complete loss of vision (Marciano-Cabral and Cabral, 2003; Lorenzo-Morales, Khan and Walochnik, 2015)

The **diagnosis** of the disease presents certain difficulties. AK is unilateral disease that progresses slowly, a diffuse superficial keratopathy may be found at the beginning of the disease and multifocal stromal infiltrates are almost always seen later so, if the diagnosis is not made at an early stage, the amoebae go into the corneal stroma and therapy becomes extremely difficult and dangerous (Lorenzo-Morales, Khan and Walochnik, 2015). Furthermore, this disease is highly confused with other types of keratitis in the early stages of infection such as atypical herpes simplex or fungal keratitis and often misdiagnosed in the first instance (Marciano-Cabral and Cabral, 2003; Ahmed *et al.*, 2022).

As for the **treatment** of AK, this has been one of the key points in the study of the disease and so far, no chemotherapeutic agent has been described as effective treatment against the malignancy (Lorenzo-Morales, Khan and Walochnik, 2015). Current treatments are characterized using antiamoebic agents, the most effective being diamidines (PHMB or chlorhexidine) and biguanides (propamidine or hexamidine). Even so, the use of these requires intensive treatment to achieve maximum trophozoite lysis by administering the drugs every hour for the first 48 hours and finally reducing the treatment every 2-3 hours for the next 4 weeks (Pérez-Irezábal et al., no date; Roberts and Henriquez, 2010). Besides, infected individuals should be followed for 6 months to ensure complete lysis of the parasite (Pérez-Irezábal et al., no date). It's seen that this treatment has some activity against the cyst stage and mainly affects the trophozoite stage, but overuse of these drugs has been found to be associated with toxic keratopathy, so a new drug for the disease is required (Roberts and Henriquez, 2010).

1.3.3. Phagocytosis and intracellular survival

Acanthamoeba feeds mainly on bacteria of the *Enterobacteriaceae* group, most notably *E. coli*, through two processes: non-specific pinocytosis and receptor-mediated phagocytosis. The mechanism of phagocytosis involves the induction of bacteria when they bind to the appropriate receptor leading to the formation of a phagosome. (**figure 4**) This is capable of binding to a lysosome for the formation of a phagolysosome, which can detect the products within it and decide whether they should be degraded or not. Finally, the enzymes of the lysosome are then able to create an acidic atmosphere which degrades the bacteria for further digestion as nutrients (Henriquez *et al.*, 2021).

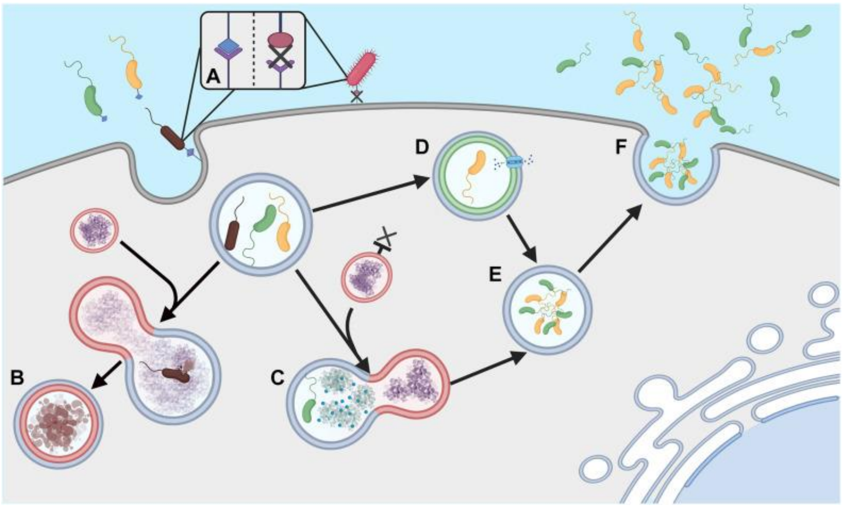


Figure 4. Phagocytosis evasion strategies. (A) Ingestion of extracellular bacteria via receptor binding. (B) phagocytosis process: the phagosome is formed and binds to the lysosome to form the phagolysosome where the bacteria will be degraded. (C) Neutralisation of lysosome enzymes for survival. (D) Formation of vacuoles or protective microenvironment. (E) Survival in the host and increased bacterial reproduction and growth. (F) Externalisation of bacteria for the infection of other amoebae (Henriquez, et *a*l 2021).

Nevertheless, studies on symbiotic relationships between unicellular eukaryotes and prokaryotes show a possible endosymbiotic relationship between the opportunistic pathogen and different bacterial species (**figure 4**). Some bacteria have been observed to avoid these procedures by preventing receptor binding and thus inhibiting this mechanism (Schulz *et al.*, 2014). Others secrete substances capable of neutralising lysosomal enzymes or prevent the formation of lysosomes through the formation of layers, creating a microenvironment that protects them and allows them to obtain nutrients from the host (Henriquez *et al.*, 2021). This evolutionary mechanism allows the bacterium to use the amoeba as a host for protection and to be released to the outside world for a more potent infection (Thomson *et al.*, 2017).

1.3.4. Endosymbiosis potential impact on monitoring and mitigation of antimicrobial resistance

This resistance mechanism has been observed in different microorganisms such as bacteria, viruses, fungus, and cyanobacteria and has a major impact on human health due to the similarity between the receptor-mediated phagocytosis mechanism present in most free-living amoebae and the signalling pathways present in human immune cells. This confirms that endosymbiotic associations within protists have a direct impact on their increased pathogenicity and an indirect impact on human health (Henriquez *et al.*, 2021).

Bacteria play an important role in AK disease causing a complex called The *Acanthamoeba* Keratitis Microbial Complex (AKMC). The main bacteria observed in AK cases are gram-negative bacteria such as *Enterobacteriaceae* (25.5% of cases) and *Pseudomonas aeruginosa* (14.1%). Gram-positive bacteria have also been observed: *Co-Straphylococcus* (15.45%), *Staphylococcus aureus* (12.8%) and *Streptococcus pneumonia* (8.72%) (Henriquez *et al.*, 2021). These use *Acanthamoeba* as a host or carrier through the mechanism of resistance to phagocytosis and once the amoebae infect the patient, they are released to the outside world increasing the infection produced (**figure 5**) (Feng *et al.*, 2009). This implies that it is one more factor to consider in the search for an adequate treatment for this disease which is in continuous evolution and shows more and more signs of AMR.

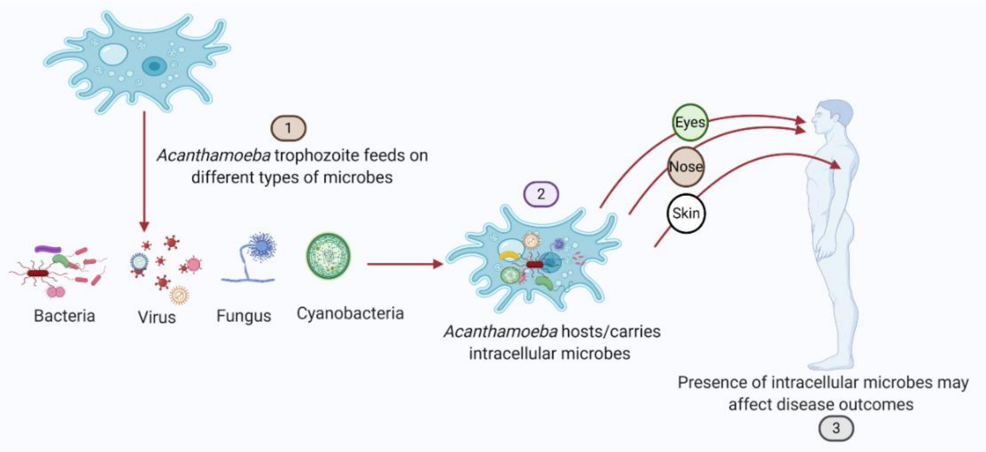


Figure 5. Endosymbiosis mechanism and the impact in AK. 1. Microorganisms interact with *Acanthamoeba*. 2. They can inhibit the phagocytosis mechanism and use the amoeba as a carrier for protection, growth, and reproduction by forming the AKMC complex. 3. *Acanthamoeba* infects the human organism, and the micro-organisms are released to the outside causing further infection (Rayamajhee, *et a*l. 2021).

Co-infectious bacteria introduced to the eye by *Acanthamoeba* can mask the AK infection, often resulting in misdiagnosis and subsequent treatment using antibiotics that are ineffective against the amoeba. Moreover, the efficacy of antibiotics against bacteria surviving intracellularly within the amoeba is currently unknown and potential exposure to sub-lethal concentrations could increase the risk of AMR emerging in patients. Thus, it is important both environmentally and clinically to understand the role that amoeba and other predatory protists play in the emergence and spread of AMR (McBride *et al.*, 2005).

1.4. Antibiotics and mode of action

The antibiotics used in this study have been selected due to their presence in the study site of a complementary work in collaboration with the Indian Institute of Technology (IIT) Bombay in Mumbai. These are Sulfamethazine, Erythromycin, Enrofloxacin and Azithromycin antibiotic and they are effective against gram-negative bacteria (Silhavy, Kahne and Walker, 2010).

Sulfadimidine or sulfamethazine (SMZ) is an antibacterial sulfonamide. This bacteriostatic anti-infective acts by blocking the biosynthesis of folic acid, which transports monocarbon units essential for the synthesis of nucleic acids. Sulphadimidine exerts a competitive inhibition of dihydropteroate synthetase (DHPS), the enzyme responsible for the incorporation of para-aminobenzoic acid for the formation of dihydropteroic acid, essential for bacterial growth. Its spectrum of action includes *Salmonella spp, Escherichia coli, Klebsiella spp, Shigella spp, Haemophilus spp, Pasteurella spp, Bordetella bronchiseptica, Fusobacterium necrophorum* all gram-negative, but some effectiveness on gram-positive as *Streptococcus spp* can also be observed (Leus *et al.*, 2022; National Center for Biotechnology Information, 2023).

Erythromycin (ERY) is an antibiotic of the macrolide family. It works by inhibiting protein synthesis in bacteria and its spectrum covers primarily gram-positive and some gram-negative bacteria. Although it is not primarily gram-negative, it has strong potential in *Legionella, Bordetella, Campylobacter* and *Helicobacter* species (Alvarez Martínez and García del Pozo, 2002).

Enrofloxacin (ENR) is a fluoroquinolone antibiotic that inhibits the enzymatic activity of bacterial gyrase and topoisomerase IV. It is active against a wide range of gram-positive and gram-negative bacteria including *Pseudomonas aeruginosa*, *Escherichia coli* and *Enterobacter*. It is also active both in the stationary phase and in the growth phase of bacterial replication (Grabowski *et al.*, 2022).

Azithromycin (AZM) is known as an azalide antibiotic structurally related to the macrolide family. The mechanism of action is based on inhibition of bacterial protein synthesis, inhibition of proinflammatory cytokine production, inhibition of neutrophil infestation, alteration of macrophage polarization and, in addition, it can enter bacterial extracellular vesicles (Heidary *et al.*, 2022). AZM is active against gram-negative bacteria as *Haemophilus*, *Morazella*, *Neisseria* and *Spirochaetal enterobacteria* and slightly less active against gram-positive *Staphylococcus aureus* and *Streptococcus* (Dey, 2022).

The Linezolid and Oxacillin antibiotics were also found in the study, but these are typically effective against gram-positive bacteria.

Linezolid (LNZ) is an antibiotic used for the treatment of infections caused by gram-positive bacteria resistant to other antibiotics. It acts mainly against streptococci, vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus*. It selectively inhibits bacterial protein synthesis through a specific mechanism of action by binding to the bacterial ribosome (23S of the 50S subunit) preventing the formation of the 70S functional initiation complex which is an essential component of the translocation process (Azzouz and Preuss, 2022).

Oxacillin (OXA) is a narrow-spectrum beta-lactam antibiotic. Its β-lactam ring covalently binds to penicillin-binding proteins, which are enzymes involved in bacterial cell wall synthesis. This binding interaction interferes with the transpeptidation reaction and inhibits the synthesis of peptidoglycan, a major component of the cell wall. By decreasing the integrity of the bacterial cell wall, it is believed to kill actively growing bacteria through cell autolysis. It acts primarily against *Staphylococcus* species bacteria which are resistant to penicillin (Castle, 2007).

2. OBJECTIVES

The main objective of the research project is to determine if antibiotic resistance in bacteria is influenced by the amoeba-bacteria endosymbiotic relationship.

To this end, there are three secondary objectives.

1. To characterise antimicrobial resistance traits of bacterial species that are intracellular to *Acanthamoeba* isolated from the environment through samples obtained via a collaborative study in India and compare them to bacteria that are extracellular to *Acanthamoeba*.
2. To determine whether *Acanthamoeba* can harbours bacteria during a *Acanthamoeba* keratitis infection.
3. To perform comparison between clinical and environmental isolates of *Acanthamoeba* and their bacteria load.

3. MATERIALS AND METHODS

3.1. Preparation of the medium

The bacterial culture of the study samples was performed on different agar plates. Lysogeny Broth (LB) agar is a widely used medium which contains adequate nutrients necessary for proper bacterial growth (MacWilliams and Liao, 2006). Mueller Hinton (MH) agar is a broadly used bacterial growth medium for antibiotic susceptibility testing with the Kirby-Bauer disc diffusion method (*Mueller Hinton Agar*, no date). Non-nutrient (NN or N) (Thermo Fisher, nd) is characterised by the absence of nutrients and highly recommended for the detection of free-living pathogenic *Acanthamoeba* species in cyst and trophozoite stages (Hardy diagnostics, nd).

The preparation of the different media was performed with the recommended concentration of LB miller large granules (Fisher chemical), MH dehydrated CM0337B (OXOID) and NN DMEM/F-12 powder agar (Thermo Fisher Scientific). It was then sterilised at 121°C for 15 minutes and finally, aliquots of the medium were transferred to the different plates next to the bunsen for their sterile maintenance and subsequent correct use.

In addition, Encystment Media (EM) and Lysogeny Broth Media (LBM) were prepared. EM is a medium that encourages the encystment process keeping the cyst state for study. For this process, 2.42 grams of Tris base was diluted in 100 mL of distilled H20. Next, the pH of the solution was tested with HCl in the pH probe (Orion versaStar PRO, ThermoScientific) until a pH of 8.8. In addition, 500 mL of distilled H20, 7.45 grams of KCl, 1.97 grams of MgSO4, 0.059 grams of CaCl2 and 0.084 of NaHCO3 were added to an Erlenmeyer flask. Then, the 100 mL pH adjusted sample was added and made up to 1000 mL with distilled H20. This solution was autoclaved at 121°C for 15 min and aliquots of solution were made in falcon tubes and stored in the refrigerator at 4°C. Respecting LBM, it is a nutrient medium for bacterial growth. It was prepared from 20 g of LB powder, which was diluted with 1 L of distilled H20. The mixture was sterilised in an autoclave at 121ºC for 15 minutes and then, the medium was transferred to small aliquots, which were kept at a temperature of 4ºC.

3.2. Environmental bacteria samples

The different procedures of environmental *Acanthamoeba* were performed on samples from India. These were collected from Vashi Creek (Mumbai) during 7/10/2021 and named as follows: S/S, S/R, S/M, S/1U, S/2U, S/1D and S/2D (**figure 6**). The initial S and present in all the samples indicate that they are sediment samples. Then, S sample indicates that it was taken from the shore, R as a reference or discharge point, M from the mangrove tree and U samples from upstream and D from downstream of the reference point.

Sampling Point	Code	Distance from Discharge Point (3) (Km)
1	S/7/10/2021	1.344
2	M/7/10/2021	1.344
3	R/7/10/2021	----
4	1U/7/10/2021	1.039
5	2U/7/10/2021	3.359
6	1D/7/10/2021	1.018
7	2D/7/10/2021	2.257

Figure 6. Vashi Creek (Mumbai, India) sampling. The image represents the different samples on the map which were collected from each location from 50 cm above the sediment surface. The table shows the number of each point, the corresponding sample, and the distance in km of the sample point from the reference point.

3.2.1. PCR of environmental samples

A PCR technique was performed for the environmental samples with Expand High Fidelity PCR System kit following the recommended instructions. The primers used were JDP1 primer (5′GGCCCAGATCGTTTACCGTGAA3’) and JDP2 (5′TCTCACAAGCTGCTAGGGAGTCA3’) which exclusively hybridises ~ 450 bp fragment of the *Acanthamoeba* 18S rRNA gene in regions E23-29 and E-23-6 (Schroeder *et al.*, 2001). In addition, the entire 16S region was used with the primers 27F (5′AGA GTT TGA TCC TGG CTC AG 3') and 1492R (5′GGT TAC CTT GTT GTT ACG 3') as a control for bacteria in the sample. Finally, the gel was read with the G: BOX gel reader (SYNGENE).

3.2.2. Extracellular isolation

The bacterial isolation protocol was divided into four procedures: Bacterial isolation and growth, Gram Staining, Antibiotic Susceptibility Test (AST) and preparation of stock samples (Annex A).

The first step was based on bacterial isolation and growth. For this, a small aliquot of each sample was taken and diluted with 10 mL of ultrapure H20. Then, a volume of 100 µL was spread on an LB agar plate which was kept under optimal growth conditions: 24 hours at 25ºC. Next, the different bacterial colonies present on the plate were identified empirically and enumerated depending on the colour and shape of the species. These were picked up with the inoculating loop and re-cultured on LB agar plates where they grew in isolation under the same optimal conditions.

The second process of the assay was Gram Staining. This procedure was performed for antibiotic selection in the following test depends on gram-negative and gram-positive bacteria results. It was added 20 µL of ultrapure H20 to a slide and each study colony was streaked with the loop. The slides were dried, and the staining process was started. The protocol followed was 1 minute of crystal violet, 1 minute of lugol's solution, use of decolorizer solution to remove the remaining crystal violet and 2 minutes of fuchsin. Besides, between each step the sample was washed with water. Then, the samples were observed under the microscope at 1000x (KYOWA, UNILUX-12) with the use of immersion oil.

The third protocol was the AST. Gram-negative colonies were treated with 4 antibiotics: SMZ 30 µg, ERY 30 µg, ENR 5 µg and AZM 15 µg. Gram-positive colonies were treated with 6 antibiotics, previously named with LZD 10 µg and OXA 1 µg.

For the test, the different study colonies were diluted with ultrapure H20 until an optical density value of 0.1 was achieved in the spectrophotometer (JENWAY Genova Plus) at 600 nm, equivalent to a concentration of $[1 \times 10^5 cells/mL]$. Next, SMZ antibiotic was needed to be prepared diluting 0.03 gr of SMZ 99 % with 1 mL of Methyl Sulfoxide (DMSO) 99.6% and adding 10 µL of the solution in a blank disc. In addition, a DMSO blank disc was made as a control to confirm the functionality of SMZ antibiotic. After that, the samples were spread on MH plates using cotton swabs in triplicate and the different antibiotic disc were placed on the plate leaving enough space between them for a better reading of the results. The plates were left in the incubator (Oribital Shaker-Incubator ES-80) at 25°C for 18-20 hours.

The last step was performed only for the plates with positive result in the AST and therefore, contain bacteria resistant to any of the antibiotics tested. For these samples, a glycerol stock was made to be able to work with them in the future. The resistant bacteria were diluted with 5 mL of LB in continuous movement for 24 hours at 25ºC. Then, 750 µL of the sample was mixed with 750 µL of glycerol > 99% and LBM. Finally, the sample was stored at -80ºC, conditions where it was kept in cryopreservation.

3.2.3. Intracellular isolation

The protocol for the intracellular bacterial isolation was divided into two main blocks. The first was based on the extraction of the bacteria from inside *Acanthamoeba* (Annex B) and the second was common with the extracellular isolation (Annex A).

Primarily, a small amount of each sample was spread with the loop in one corner of an NN agar plate. It was then left at room temperature and viewed under the inverted microscope (LEICA DMI1) until an area with *Acanthamoeba* was identified. Subsequently, the amoeba agar fragment was marked, cut with a scalpel, and placed on the top fragment of a new NN plate. After that, 40 µL of dead *E. coli* was added in the central strip, as it is the main nutrient for the amoeba. This was done to allow the amoeba to move to the bottom fragment of the plate where it would be completely isolated. The plates were then left at room temperature and the progress of the amoeba was observed every 48 hours.

Once the presence of isolated amoebae was identified, the agar slice was marked, cut, and transferred to a 24 well-plate by covering the agar with EM, which induces the encystment procedure by reducing the metabolic state of the amoeba. This new well plate was kept for 48 hours at room temperature. The presence of *Acanthamoeba* in each of the test wells was then checked under the inverted microscope (LEICA DMI1) and transferred to a new 24-well plate where the name of each sample was written in duplicate (A and B). The agar from the previous plate was run into well A and covered with 1 mL of EM. The remaining medium was removed by pipette to suspend the amoebae and 300 µL of the sample was collected and with 700 µL of EM was transferred to well B. This well A was left as a reserve and well B for future study. The plates were kept for 24-72 hours at room temperature.

Finally, well B was removed with the help of a pipette, and with a 27G needle, the medium was absorbed and discarded in the same well. The needle has a hole large enough for the bacteria to pass through, but small for the amoeba, causing its fragmentation. The purpose of the procedure was to allow bacteria, that were present intracellularly, to pass into suspension in the medium.

The second block of the practical is in common with extracellular isolation. For this, 100 µL were removed from the homogenised well B and spread on an LB agar plate. The same procedures were then followed as for the extracellular: Bacterial isolation and growth, Gram Staining, AST, and preparation of stock samples.

3.3. Minimal Inhibitory Concentration test

The Minimal Inhibitory Concentration (MIC) test is defined as the level of resistance of a specific bacterium to an antibiotic expressed in μg/mL (Kowalska-Krochmal and Dudek-Wicher, 2021). The antibiotic tested was SMZ and the bacteria of study were *E. coli*, due to the high concentration of this in the samples; and *Ps. aeruginosa* and *Ps. putida M. cherry*, as there is controversy about the functionality of SMZ in *Pseudomonas* and due to the high concentration of these in clinical cases of AK (Abd El-Ghany, 2021; Ovung and Bhattacharyya, 2021). In addition, *Acanthamoeba castellanii* ATCC 50370 (USA) was used as study amoeba due to its high pathogenicity in AK disease (ATTC, nd).

3.3.1. Determination of MIC in bacteria

The protocol followed started by introducing the study bacterial colony in LBM to make it growth. Then, an aliquot was taken, and the optical density was measured until have a value of 0.1, equivalent to $10^9\ cells/mL$ for *E. coli* and *Ps. aeruginosa* and $10^8\ cell/mL$ for *Ps. putida*. The final bacterial study concentration was $2 \times 10^5 cells/mL$ in a final volume of 4 mL. For this, the necessary sample volume was calculated to achieve the study volume and concentration.

On the other hand, the antibiotic and control were prepared. The SMZ had a final concentration of 5.00×10^4 μg/mL and for this, 50 mg of SMZ 99% was diluted with 1 mL of DMSO 99.6%. Next, serial dilutions of the antibiotic and control were performed with LB until a concentration of 2.50×10^1 μg/mL was achieved.

Following, the bacteria and the SMZ or DMSO dilutions made were load to a 96-well plate (**figure 7**). For each bacteria of study, two plates were made (one for SMZ and one for DMSO) and the wells were loaded in triplicate to obtain more accurate results. The first column was for the control, and it was loaded with 50 μL of LB and 50 μL of the study bacteria in the first three wells and 100 μL of LB in the three last ones. The second column represents the maximum concentration, so 50 μL SMZ [50 mg/mL] or 50 μL DMSO 99.6% were loaded with 50 μL of LB. From column 3 to 12 the different dilutions were loaded; the first three wells of the column were loaded with 50 μL of the corresponding dilution and 50 μL of the bacteria; and the last three wells were loaded with 50 μL of the dilution and 50 μL of LB.

Figure 7. Representation of the 96-well plate used in the test and the volumes used for each well. Created by Biorender.

Finally, the well-plates were left for 24 hours at 37 °C and a subsequent reading of the optical density of the wells at 595 nm was performed in the absorbance 96 Plate Reader (Infinite F200 PRO). This practice was performed in triplicate to obtain more accurate results.

3.3.2. Determination of MIC in *Acanthamoeba*

The MIC test for SMZ in *A. castellanii* ATCC 50370 was performed following the alamarBlue reduction assay. For this purpose, a sample of the amoeba was taken and put it in PAGE media up to (90-95) % of confluence. Then, it was diluted $1/10$ and 10 µL of the dilution was placed in a Neubauer chamber for cell counting in the inverted microscope (LEICA DMI1). This was done in duplicate, and the results were averaged. From the cell counting results, the required cells/mL concentration was calculated and determined.

$$\frac{cell}{mL} = cell\ count\ \times dil.factor \times 10^4 cells/mL$$

Next, calculations were then made to determine the volume required to obtain an amoeba concentration of $4 \times 10^5\ cells/mL$ in a final volume of 4 mL.

The last step was to load two 96-well plates, one for SMZ and one for DMSO with the same pattern as for the bacterial MIC (**figure 7**). The wells were loaded in triplicate to obtain more accurate results. For this, 50 µL of PAGE medium was loaded into the columns corresponding to the control and the different dilutions, leaving the second column free. Next, 100 µL of DMSO 99.6% or SMZ 99% were loaded into the 6 study wells of column 2. Then, 50 µL of these were taken and added to column 3 corresponding to the first dilution, 50 µL of this was taken and added to column 4, following this pattern until the last column, thus making serial dilutions of the SMZ and DMSO. Next, 50 µL of PAGE medium was loaded into the last three wells of each column and 50 µL of the $4 \times 10^5 cells/mL$ amoeba solution into the first three wells. Finally, 10 µL of alamarBlue TM Cell Viability Reagent (Invitrogen, Thermo Fisher Scientific) was added to each of the study wells and the plate was left for 24 hours at 25 °C. The optical density of the wells was then read in the absorbance 96 Plate Reader (Infinite F200 PRO) at 570 and 595 nm. This practice was also performed in triplicate to obtain more accurate results.

After that, the absorbance results were collected and the percentage reduction of alamarBlue was calculated to determine the viability of the amoeba through the following formula.

$$(\{[(\varepsilon\mathrm{ox}\,\lambda_2)(\mathrm{A}\,\lambda_1)] - [(\varepsilon\mathrm{ox}\,\lambda_1)(\mathrm{A}\,\lambda_2)]\}/\{[(\varepsilon\mathrm{red}\,\lambda_1)(\mathrm{A}'\,\lambda_2)] - [(\varepsilon\mathrm{red}\,\lambda_2)(\mathrm{A}'\,\lambda_1)]\}) \times 100$$

The value of εred λ_1 is 155,677 (molar extinction coefficient of reduced alamarBlue at 570 nm); εred λ_2 is 14,652 (molar extinction coefficient of reduced alamarBlue at 600 nm); εox λ_1 is 80,586 (molar extinction coefficient of oxidized alamarBlue at 570 nm); εox λ_2 is 117,216 (molar extinction coefficient of oxidized alamarBlue at 600 nm); A λ_1 is the absorbance of the test wells at 570 nm; A λ_2 is the absorbance of the test wells at 600 nm; A' λ_1 is the absorbance at 570 nm of the negative control wells, which contained medium and alamarBlue but to which *Acanthamoeba* was not added; and A' λ_2 is the absorbance at 600 nm of the negative control wells, which contained medium and alamarBlue but to which *Acanthamoeba* was not added (McBride *et al.*, 2005).

3.3.3. Determination of MIC in co-cultures of bacteria and *Acanthamoeba*

The last MIC test was conducted to compare the effect of the antibiotic on bacteria, amoeba and both species coexisting to observe the endosymbiotic effect that may exist. The protocol followed was bacterial and amoeba growth and dilutions as the same done for bacteria and amoeba MIC. Then, three SMZ concentrations were chosen based on the bacterial MIC test results corresponding to high, medium, and low antibiotic concentration where the last one corresponded to the MIC of each study bacteria. Lastly, the different dilutions were loaded intro two 24-well plates for each bacterium following the pattern shown in **figure 8**. The amoeba was first added with PG to extend in the media for 30 minutes, then the corresponding volume of bacteria was added so that the species could interact between them for one hour. Finally, the volume of antibiotic required for each concentration was calculated and added for a final volume of 1 mL.

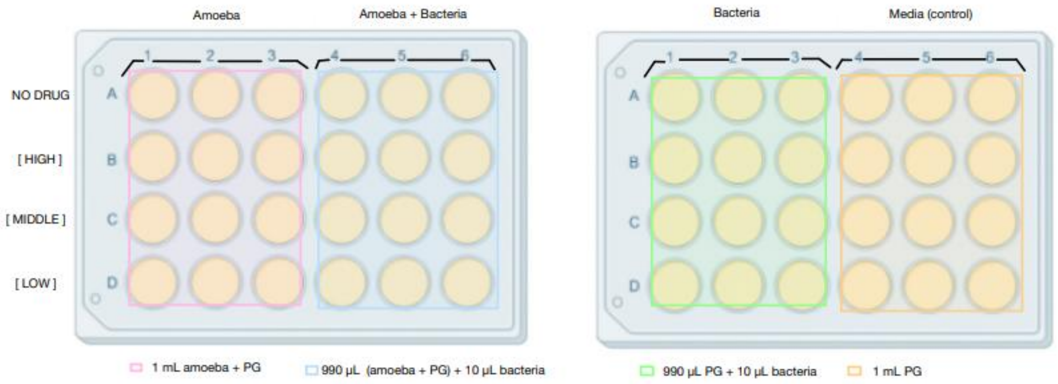


Figure 8. Representation of the 24-well plate used in the test and the volumes used for each well. Created by Biorender.

Next, the plates were left for 18-20 hours at 25 °C and then, the medium was removed and 1mL of LB was added to all wells and were left at 37 ºC for 24 hours. It was done to remove the antibiotics and add a nutrient-enriched medium for the bacteria. Finally, the optical density of the wells was read at 595 nm in the absorbance 24 Plate Reader (Infinite F200 PRO). This practice was done by triplicate.

3.4. Are bacterial present in *Acanthamoeba* clinical isolates?

Acanthamoeba samples isolated from AK patients were provided by Richard Childs Hunt, a PhD student at the London School of Hygiene and Tropical Medicine (London, UK).

3.4.1. DNA extraction and DNA amplification of clinical samples

The confirmation of the presence of amoebae and bacteria in the samples was carried out by PCR of the samples. For this purpose, a DNA extraction was first executed using the QIAamp R fast DNA stool mini kit according to the manufacturer's instructions for human samples. Finally, the DNA concentration of the samples was measured in ng/µL on the Qubit^TM4 fluorometer (Invitrogen ThermoFisher Scientific).

The PCR technique was performed for same primers used in the environmental samples: JDP and 16S. The amplification reaction for JDP was performed with Expand High Fidelity PCR System kit following the recommended instructions; however, for 16S the whole region, 2x Bio Mix Red 500 reactions were used following the established indications. Finally, the gels were read with the G: BOX gel reader (SYNGENE).

4. RESULTS

4.1. PCR *Acanthamoeba* in environmental samples

The first results of the project were obtained for PCR of environmental samples to confirm the presence of *Acanthamoeba* in these study samples.

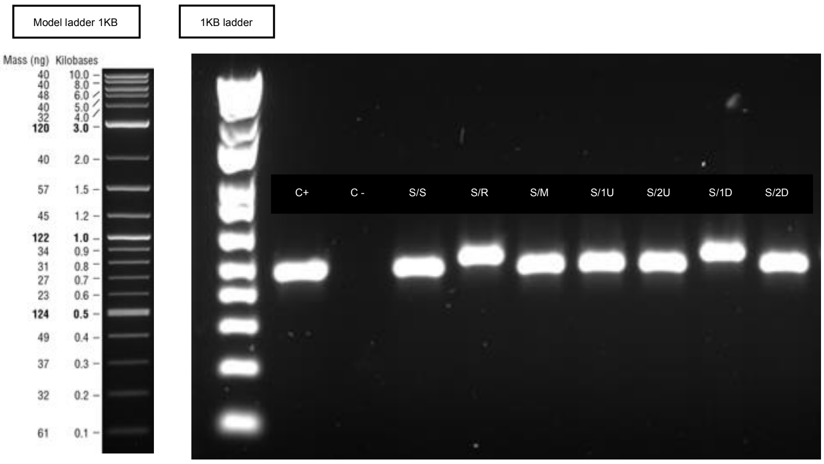


Figure 9. PCR environmental samples for JDP primer. The first column corresponds to the 1kb Plus DNA ladder model used for the experiment with the number of kilobases and mass in ng for each of the strips. The second column corresponds to the load of the ladder used. The third and fourth columns correspond to the positive (*A.castellanii*) and negative control respectively. The rest of the columns correspond to the seven study samples.

The PCR for the environmental samples from Vashi Creek (Mumbai, India) (**figure 9**) showed positive results for the JDP primer between the 0.4 and 0.5 kilobase bands. This result confirmed the presence of *Acanthamoeba* in all the samples and therefore confirm their use for the study.

4.2. Antibiotic Susceptibility Test

The results of the AST were then analysed for bacteria isolated extracellularly and intracellularly from amoeba.

For the analysis of these results, first the halo from each antibiotic was measured, which corresponds to the inhibition diameter presented by each bacterial colony. Next, the average of the replication carried out for the practice was calculated and the values obtained for the extracellular and intracellular samples were compiled in different tables shown in Annex C and Annex D, respectively. The different tables present in the first column the samples tested with a colony number, which was determined by colour and shape differentiation. The other columns represent the antibiotics under study and the control: SMZ, ERY, ENR, AZM, LZD, OXA and DMSO with the mean of the results of the inhibition halo diameter of each bacterial colony. Values with a result of 0.333 have been rounded to 0.3 and values of 0.666 have been rounded to 0.7.

Analysing the results of AST results for bacteria isolated from the outside of the amoeba (Annex C) highlighted the presence of a double halo for samples S/2U (colony 2 and 4) and S/M (colony 1) and therefore only the inhibition halo corresponding to the outer halo was collected in the results. In addition, a small halo was observed for the control (DMSO); however, the SMZ values of the respective samples had values above 30 mm and were considered valid. Regarding the results for intracellular isolation bacteria (Annex D), there were no results for two study samples: S/S and S/1D. This was because an aliquot containing *Acanthamoeba* could not be isolated during the internship period, and therefore no analysis was performed for these.

Once the inhibition values were obtained, the criteria for determining whether the test was positive or negative was based on the reference compilation values for AMR in different bacterial species from the EUCAST, CLSI and BSAC databases (Annex E). The table shows the six antibiotics tested, the bacterial species compiled from the databases as a guide and the minimum halo diameter values in millimetres to determine whether the bacterium is resistant to the antibiotic under study. Finally, from these values, positive results to the test antibiotics were marked in red and positive results to DMSO were marked in blue in the tables in Annex C and Annex D.

4.3. Multiple Antibiotic Resistance Index

Based on the analysis performed for the AST, the possible multiple resistance of bacteria to antibiotics was tested using the Multiple Antibiotic Resistance (MAR) index. For this purpose, the samples used were grouped into three main groups according to the geolocation of the river: upstream (S/1U and S/2U), middle (S/S, S/M and S/R) and downstream (S/1D and S/2D).

The MAR index was calculated and interpreted according to Krumperman (1983) using the formula $\frac{a}{(b \times c)}$ where ‘a’ represents the number of antibiotics to which an isolate was resistant, ‘b’ represents the number of isolated bacteria in the sample and ‘c’ represents the number of antibiotics tested. It was determined that values of MAR greater than 0.2 indicates a high-risk source of contamination where antibiotics are often used (Ayandele *et al.*, 2020).

$$MAR\ INDEX = \frac{N^{\underline{o}}\ of\ antibiotics\ to\ which\ an\ isolate\ was\ resistant}{N^{\underline{o}}\ isolated\ bacteria\ in\ the\ sample\ \times N^{\underline{o}}\ of\ antibiotics\ tested}$$

After that, the extracellular and intracellular MAR index results were plotted for the different river reaches; upstream, middle, and downstream (**figure 10**).

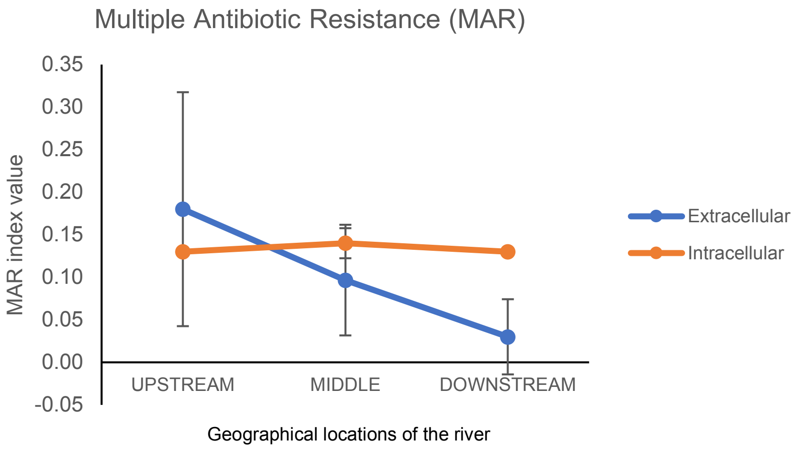


Figure 10. MAR index of extracellular and intracellular isolated bacteria. The graph represents the MAR index value (Y-axis) of extracellular and intracellular bacteria in different geographical locations (upstream, middle, and downstream) (X-axis).

The graph showed the MAR index value at the different study points for bacteria isolated from outside and inside the amoeba. The line corresponding to the extracellular bacteria showed a gradual descending line from a value of 0.18 for upstream to 0.03 for downstream; however, the line corresponding to bacteria isolated from inside the amoeba showed a constant MAR index value of 0.14 throughout the river course.

In addition, statistical analysis of the samples was performed for each of the seven study samples. A difference in results was observed in the upstream group for both extracellular and intracellular samples. However, in the middle and downstream groups the difference was minimal.

4.4. Extracellular and intracellular bacteria results

Once the multiple extracellular and intracellular resistance analysis was done for the different locations of Vashi Creek, the bacterial resistance analysis was performed for each of the antibiotics under study (**figure 11**).

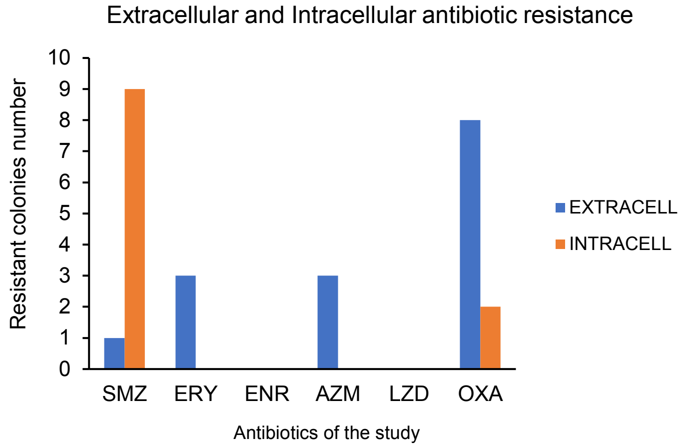


Figure 11. Number of extracellular and intracellular bacterial colonies resistant to each of the antibiotics under study. The X-axis shows the different antibiotics tested and the Y-axis shows the number of resistant bacterial colonies obtained.

The results showed a higher number of bacteria resistant to the antibiotic OXA, lower resistance to ERY, AZM and SMZ and no resistance to ENR and LZD for extracellular bacteria. However, high resistance to SMZ, lower resistance to OXA and no resistance to ERY, ENR, AZM and LZD were obtained for intracellular bacteria.

4.5. PCR bacteria in environmental samples

Finally, a PCR was performed for bacterial colonies that tested positive for AST and were therefore considered resistant to at least one of the target antibiotics (**figure 12**). This assay was performed to confirm the presence of bacteria in glycerol stock and to be able to send these samples for sequencing and to determine the bacteria species involved.

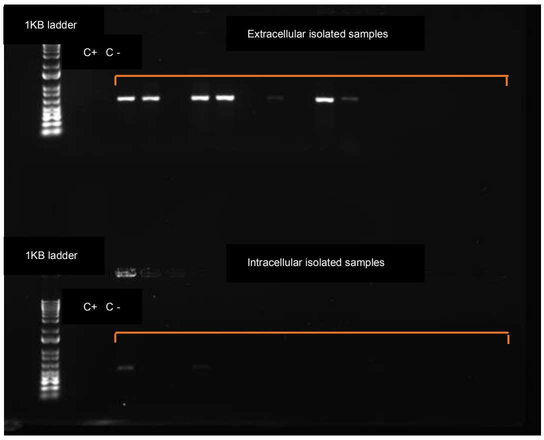


Figure 12. PCR environmental samples with 16S primer. The first column at the top and at the bottom represents 1kb Plus DNA ladder. The second and third columns represent the positive and negative control respectively.Then, the columns at the top represent extracellular samples and the columns at the bottom represent intracellular samples.

The top part of the figure is showed the results for bacteria isolated from the outside of the amoeba in the following order: S/S 5, S/R 1, S/R 3, S/M 1, S/1U 2, S/1U 4, S/2U 2, S/2U 3, S/2U 4, S/1D 5 and S/1D 6. At the bottom, samples corresponding to bacteria isolated from the interior of *Acanthamoeba* were loaded in the following order: S/R 1, S/R 2, S/R 4, S/M 2, S/M 3, S/1U 3, S/1U 4, S/2U 2, S/2U 4, S/2D 2 and S/2D 3.

These results showed the presence of bacteria to a greater or lesser extent; however, there was no result for the positive control, so this test should be repeated to obtain accurate results.

4.6. Minimal Inhibitory Concentration test

The MIC results for SMZ were then analysed for bacteria (*E. coli*, *Ps. aeruginosa* and *putida*), amoebae (*A. castellanii*) and both in coexistence. This test was performed only for one antibiotic based on the analyses performed for the MAR index results corresponding to the amoeba-bacteria endosymbiotic hypothesis and the results of the number of intracellular bacteria resistant to each antibiotic.

4.6.1. Determination of MIC in bacteria

The MIC results were plotted on a lineal graph from the optical density (OD) values (595nm) obtained for each study concentration in SMZ and DMSO. The OD values of 0 corresponded to non-living bacteria and maximum OD point correspond to 100% of the bacteria present with life.

4.6.1.1. *Escherichia coli*

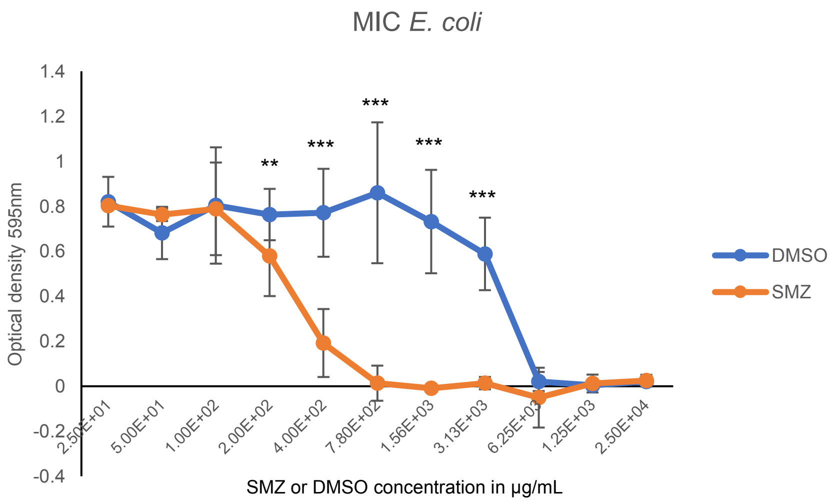


Figure 13. Linear representation of *E. coli* MIC test results. The X-axis represents the concentration of SMZ and DMSO used in µg/mL. The Y-axis represents the optical density at 595 nm for DMSO and SMZ. After T test analysis: ** P ≤ 0.01, *** P ≤ 0.001 compared to the control (DMSO).

The analysis of **figure 13** shows that DMSO presented an OD value around 0.8 for the concentration range $(2.50 \times 10^1 - 7.80 \times 10^2)$ µg/mL, where the maximum point was at 7.80×10^2 µg/mL. Thereafter, there was a decrease until the OD value of 0 corresponding to the concentration range between $(6.25 \times 10^3 - 2.50 \times 10^4)$ µg/mL. Therefore, it was observed that DMSO had a 100% effect at concentrations above 6.25×10^3 µg/mL, had a 50% effect at a concentration of approximately 4.00×10^3 µg/mL and a 0% effect at a concentration below 7.80×10^2 µg/mL for *Escherichia coli*.

Looking at the line corresponding to the antibiotic, it was observed that between 2.50×10^1 µg/mL and 1.00×10^2 µg/mL it presented an OD value around 0.8. Then, the decrease stranded out between the range $(1.00 \times 10^2 - 7.80 \times 10^2)$ µg/mL and finally, there was a value 0 for the concentration between 7.80×10^2 µg/mL and 2.50×10^4 µg/mL where the concentration of 7.80×10^2 µg/mL was determined as MIC. Hence, contrasting the results obtained with the control antibiotic assay (DMSO), it was determined that SMZ had a 100% effect at concentrations between $(7.80 \times 10^2$ and $6.25 \times 10^3)$ µg/mL where 7.80×10^2 µg/mL was the MIC on *E. coli*. At concentrations higher than 6.25×10^3 µg/mL, the effect of SMZ could not be confirmed because it could be influenced by DMSO. Then, it was seen that SMZ had a 50% effect at a concentration of approximately 3.00×10^2 µg /mL and a 0% effect at a concentration below 1.00×10^2 µg /mL.

Finally, the graph shows the significant difference presented between the antibiotic and the control for the points corresponding to the concentration range from 1.00×10^2 µg /mL to 3.13×10^3 µg /mL.

4.6.1.2. *Pseudomonas aeruginosa*

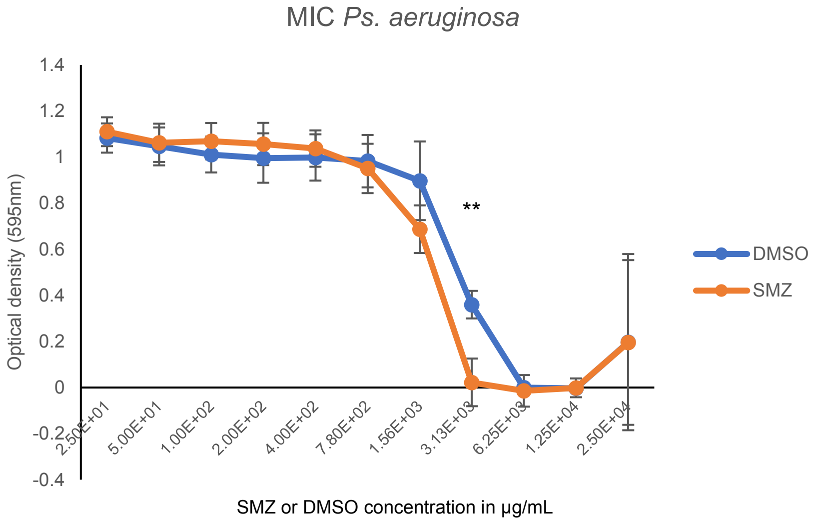


Figure 14. Linear representation of *Ps. aeruginosa* MIC test results. The X-axis represents the concentration of SMZ and DMSO used in µg/mL. The Y-axis represents the optical density at 595 nm for DMSO and SMZ. After T test analysis: ** P ≤ 0.01 compared to the control (DMSO).

Following the same pattern of analysis performed for the *E. coli* results, it is observed in **figure 14** that it presented a value of approximately 1 for DMSO between $(2.50 \times 10^1 - 1.56 \times 10^3)$ µg/mL concentration. Then, this value dropped to an OD value of 0 in the range of $(6.25 \times 10^3 - 1.25 \times 10^4)$ µg /mL. This value was not observed for the first dilution $(2.50 \times 10^4$ µg /mL) so it was determined that the value obtained in this corresponded to a pipetting or machine error in the OD measurement. Thus, it was determined that DMSO had a 100% effect at concentrations above 6.25×10^3 µg/mL, a 50% effect at a concentration of approximately 3.20×10^3 µg/mL and a 0% effect at a concentration below 1.56×10^3 µg/mL for *Ps. aeruginosa*.

Looking at the SMZ line graph, OD values around 1 were shown for concentrations of $(2.50 \times 10^1 - 7.80 \times 10^2)$ µg/mL. Next, it gradually decreased between the concentration $(7.80 \times 10^2 - 3.13 \times 10^3)$ µg/mL and finally remained stable at OD 0 for the concentration range $(3.13 \times 10^3 - 1.25 \times 10^4)$ µg/mL where 3.13×10^3 µg/mL corresponded to MIC. This value was also not observed in the first dilution as for DMSO, so an error was determined. From the contrast with the values for DMSO, it was determined that SMZ had a 100% effect on *Ps. aeruginosa* at concentrations between $(3.13 \times 10^3 - 6.25 \times 10^3)$ µg/mL. This range was for the difference between a dilution and therefore it can be seen in the graph that the lines representing the antibiotic and control are similar and therefore only one point of significant difference. Next, it was observed then that it had 50% effect at approximately 2.40×10^3 µg/mL and a 0% effect at a concentration below 7.80×10^2 µg/mL.

4.6.1.3. *Pseudomonas putida*

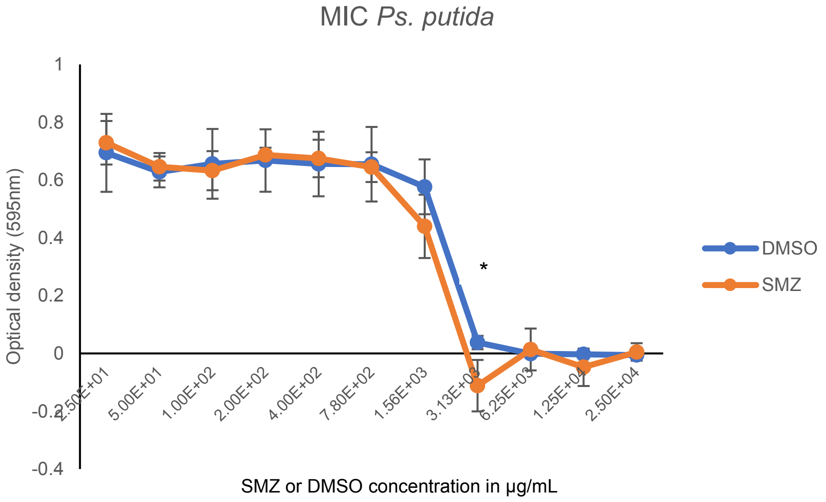


Figure 15. Linear representation of *Ps. putida* MIC test results. The X-axis represents the concentration of SMZ and DMSO used in µg/mL. The Y-axis represents the optical density at 595 nm for DMSO and SMZ. After T test analysis: * P ≤ 0.05 compared to the control (DMSO).

In the analysis of the results for *Ps. putida* (**figure 15**) both, DMSO and SMZ, showed very similar results and therefore the significant difference between the two was only one point.

This expressed an OD around 0.7 for DMSO and SMZ between 2.50×10^1 µg/mL and 7.80×10^2 µg/mL. Subsequently, this value dropped to 0 where it was maintained for the concentration range $(3.13 \times 10^3 - 2.50 \times 10^4)$ µg/mL. In addition, it was found that, at the concentration of 3.13×10^3 µg/mL and 1.25×10^4 µg/mL of SMZ, the OD value was negative which might be due to pipetting or spectrophotometer reading errors. Thus, it was determined that DMSO had a 100% effect at concentrations above 3.13×10^3 µg/mL, a 50% effect at a concentration of approximately 1.56×10^3 µg/mL and a 0% effect at a concentration below 7.80×10^2 µg/mL for *Ps. putida*. The effect of SMZ could not be confirmed, as the similarity of results does not allow to confirm that it is due to SMZ and not to the control.

4.6.2. Determination of MIC in *Acanthamoeba*

The determination of MIC in *Acanthamoeba* was represented by a line graph for the viability of the amoeba with respect to the concentration of SMZ and DMSO tested.

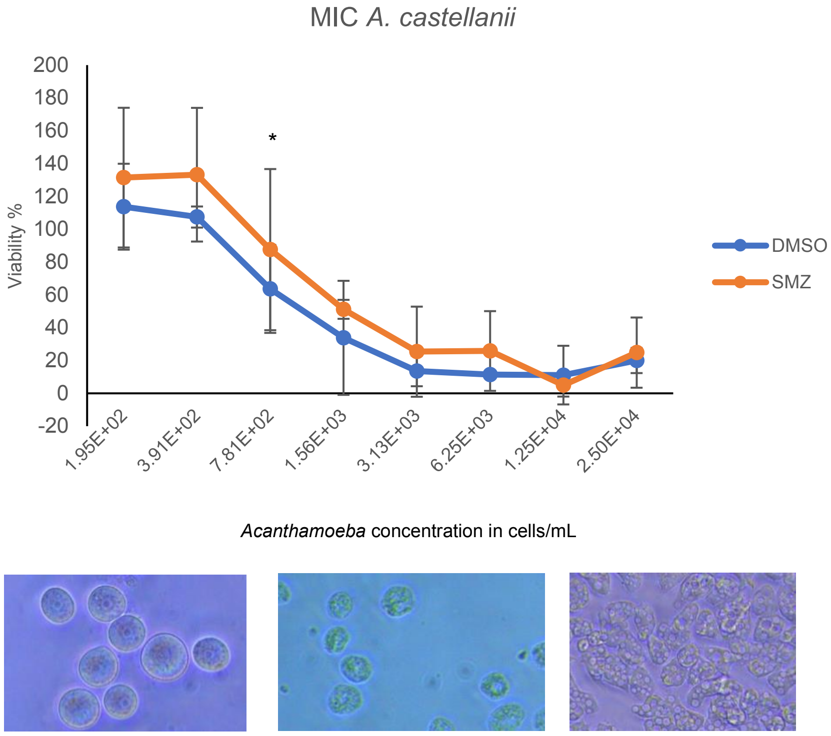


Figure 16. Linear representation of *Acanthamoeba* MIC test results. The X-axis represents the *Acanthamoeba* concentration (cells/mL) used in the test. The Y-axis represents the percentage of viability. The images below represent the state of *Acanthamoeba* at the different concentrations. After T test analysis: * P ≤ 0.05 compared to the control (DMSO).

From **figure 16** it is determined that amoeba showed a viability of 115% between the concentration range $1.95 \times 10^2\ cells/mL$ and $3.91 \times 10^2\ cells/mL$ for DMSO. This then, decreased proportionally with increasing concentration until a viability result of 20% was achieved, which was maintained for concentrations between $(3.13 \times 10^3 - 2.5 \times 10^4)\ cells/mL$. In the case of SMZ, a higher viability of 130% was reached for the $1.95 \times 10^2\ cells/mL$ amoeba concentration. Thereafter, the viability progressively decreased to 5-10% for the $1.25 \times 10^4\ cells/mL$ concentration.

From the analysis of the results, it was observed that DMSO (control) had a greater impact on *Acanthamoeba* than SMZ (antibiotic). However, the significant difference in the values obtained was minimal and therefore, it was determined that this could have been due to a chemical reaction between alamarBlue and DMSO. In addition, the results obtained for the concentration range between $(2.50 \times 10^4 - 2.00 \times 10^5)\ cells/mL$ were not plotted, as the values were well outside the expected range. This could be due to pipetting errors, exposure of the alamarBlue to light or errors in reading the results. Finally, it was determined that to obtain true results, this practice should be repeated.

4.6.3. Determination of MIC in co-cultures of bacteria and *Acanthamoeba*

The MIC results for co-cultures of bacteria and *Acanthamoeba* were plotted in a bar graph comparing the optical density value at 595 nm for the control (medium), the different study bacteria (*E coli*, *Ps, aeruginosa* and *Ps. putida*), the amoeba (*A. castellanii*) and these both in co-culture. The analysis of the significant value of the samples was performed only for the bacterial and both samples, as the aim of the practice was to study the possible increase of resistance in the bacteria when they were together with amoebae at high drug concentrations than when the amoeba was absent.

4.6.3.1. *E. coli and A. castellanii*

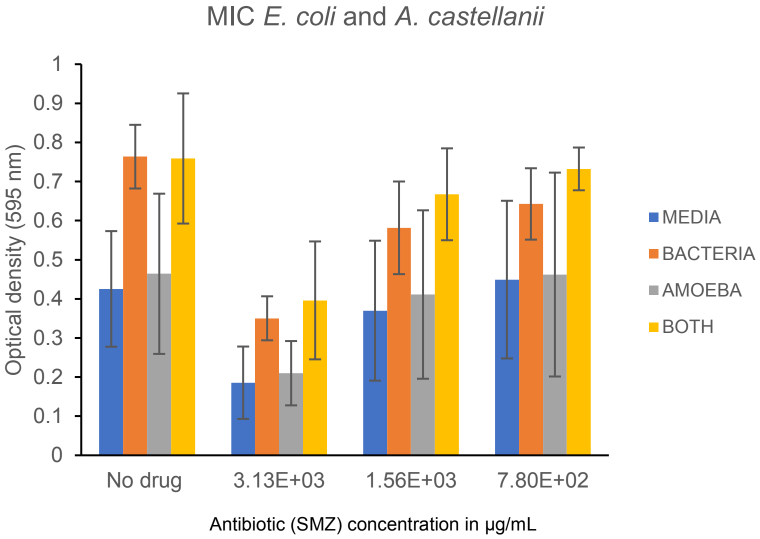


Figure 17. Bar graph representation of MIC *E. coli* and *A. castellani* results. The X-axis represents the control and the different SMZ concentrations studied (high, medium, and low) in µg/mL. The Y-axis represents the optical density for the three study standards (bacteria, amoeba, both) and the control (media).

The analysis of the MIC result for the co-culture of *E. coli* and *A. castellanii* (**figure 17**) showed first the presence of contamination in the samples. This was verified by observing the high optical density present in the control samples corresponding to the medium. However, this was only for the first triplicate and as the rest of the samples presented similar values, providing a high standard deviation in the samples, they were considered valid values.

Next, the patterns of amoebae and bacteria were analysed, where the presence of both species in the study was verified through the optical density result. In addition, it was observed how they presented a higher optical density directly related to the concentration of the species in the well corresponding to no drug, this decreased progressively in low concentration, medium and finally its lowest concentration was represented in high concentration.

Finally, the graph was analysed focusing on the bacterial and bacteria + amoeba patterns, which are related to the endosymbiotic hypothesis of the study, showing that both patterns were the same for the drug-free well. However, this changed depending on the concentration of drug used. At low concentrations (7.80×10^2 µg/mL) the difference was smaller, and the difference increased directly proportional to the drug concentration. Therefore, it was observed that at high drug concentrations corresponding to 3.13×10^3 µg/mL the concentration of amoeba with bacteria was higher than that of bacteria alone. However, the difference between the two samples was not significant.

4.6.3.2. *Ps. aeruginosa and A. castellanii*

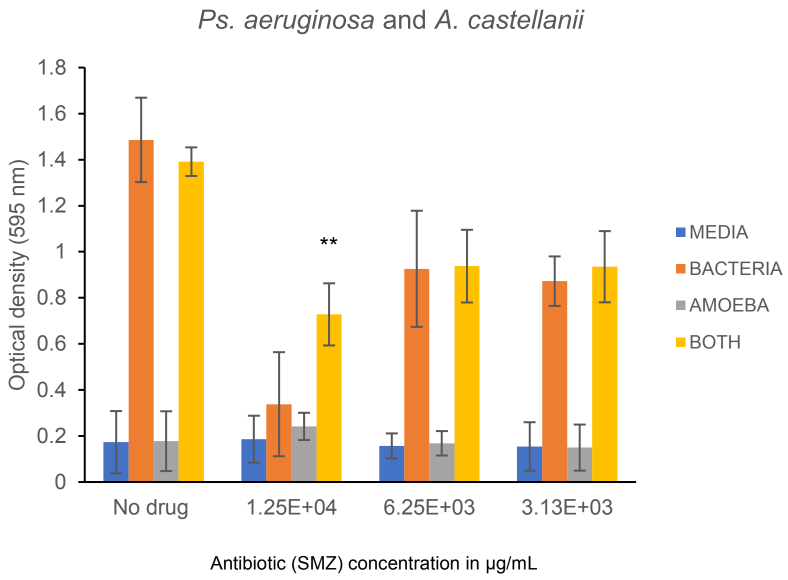


Figure 18. Bar graph representation of MIC *Ps. aeruginosa* and *A. castellani* results. The X-axis represents the control and the different SMZ concentrations studied (high, medium, and low) in µg/mL. The Y-axis represents the optical density for the three study standards (bacteria, amoeba, both) and the control (media). After T test analysis: ** P ≤ 0.01 compared to the control (DMSO).

The results for *Pseudomonas aeruginosa* and *A. castellani* (**figure 18**) showed low optical density values for the medium, confirming that the samples were not contaminated and therefore considered valid for the assay analysis.

For the bacterial and amoeba parameters, the presence of bacteria and amoeba was observed for the different study wells. Finally, the concentration of bacteria and bacteria together with amoeba was analysed. This showed a high concentration in the absence of drug, but these values decreased as the drug concentration increased, presenting almost identical concentration values for both parameters. However, for the study of these at the highest drug concentration (1.25×10^4 µg/mL) a significant difference was observed where the parameter corresponding to the bacteria with amoeba showed a higher optical density value than for bacteria alone and therefore there was a higher concentration of microorganisms in it.

4.6.3.3. *Ps. putida and A. castellanii*

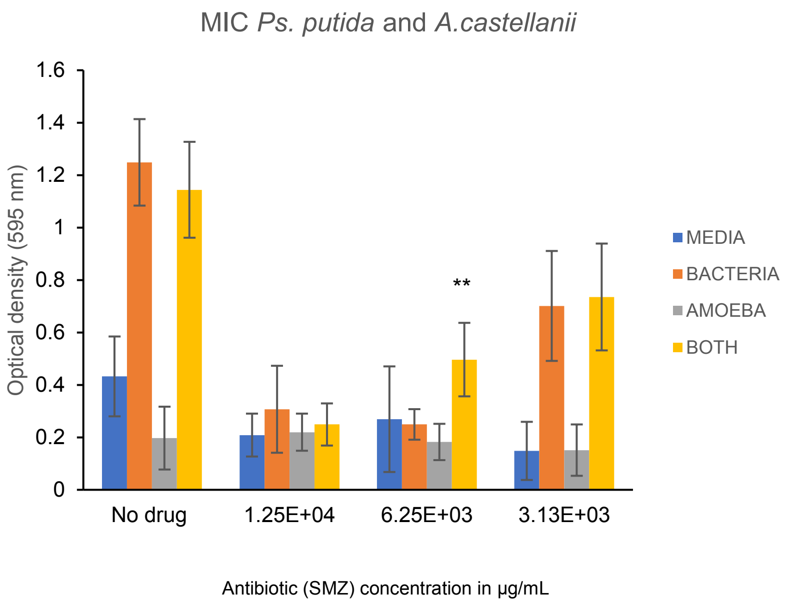


Figure 19. Bar graph representation of MIC *Ps. putida* and *A. castellani* results. The X-axis represents the control and the different SMZ concentrations studied (high, medium, and low) in µg/mL. The Y-axis represents the optical density for the three study standards (bacteria, amoeba, both) and the control (media). After T test analysis: ** P ≤ 0.01 compared to the control (DMSO).

The control result for the MIC study of *Ps. putida* and *A. castellanii* (**figure 19**) showed low optical density values for the standards corresponding to the different drug concentrations; but it showed a higher value for the non-drug wells and therefore it was determined that these wells were contaminated. However, as in the *E. coli* assay, this was only shown in one of the triplicates and therefore the values present were considered valid.

The amoeba and bacteria parameters were then analysed, where the presence of amoeba and bacteria in the different wells was verified by means of optical density values. The concentration of bacteria was higher in the absence of drug and decreased for a drug concentration of 3.13×10^3 µg/mL. However, the decrease of this was not progressive and its minimum point of concentration was for the medium drug concentration (6.25×10^3 µg/mL) and then it increased. As for amoeba, a higher concentration was observed at the 1.25×10^4 µg/mL drug concentration than in the absence of the drug. These results may be due to asserted contamination or pipetting errors as well as machine errors in the absorbance reading.

In the analysis of the bacteria and bacteria plus amoeba parameters, a high concentration of amoeba was observed in the absence of drug and a progressive decrease directly proportional to the increase in drug concentration. At a drug concentration of 3.13×10^3 µg/mL, both parameters showed a similar optical density; at a median concentration of 6.25×10^3 µg/mL, a significant difference was observed between the parameters where a higher concentration of amoeba plus bacteria was shown. However, this pattern was not repeated for the higher drug concentration of 1.25×10^4 µg/mL.

4.7. PCR Clinical samples

Finally, the clinical study of samples with *Acanthamoeba* was performed to confirm the presence of the studied amoeba and bacteria in AK patients and the importance of the coexistence of these microorganisms for an efficient treatment of the disease.

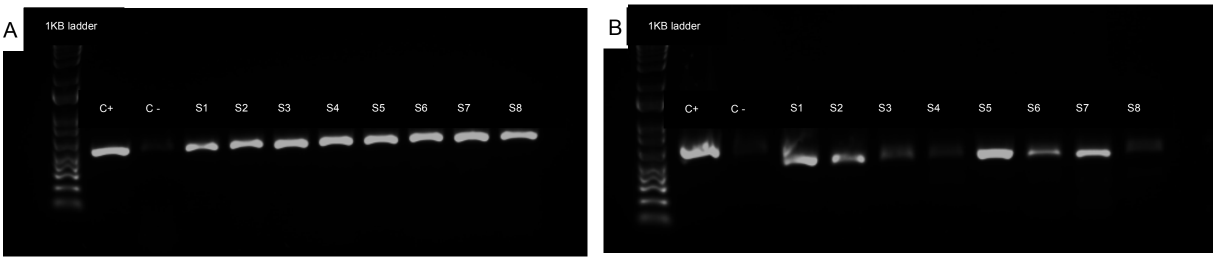


Figure 20. PCR clinical samples for JDP and 16S primers. Image A corresponds to samples treated with JDP primer; image B corresponds to samples treated with 16s primer. The first column shows 1kb Plus DNA ladder. The second and third columns correspond to the positive and negative control respectively. The rest of the columns correspond to the clinical study samples (S) of AK pacients.

The PCR results for clinical samples from AK patients were positive for the JDP primer showing a band corresponding to 0.45 bp (**figure 20**). Therefore, it confirmed the presence of *Acanthamoeba* sp. in these samples. It was also positive for the 16S primer confirming bacterial presence in the different clinical samples from AK patients.

5. DISCUSSION AND CONCLUSION

The overproduction of antibiotics mainly in LMIC countries like India has triggered one of the biggest pollution problems in the world. Analysis of natural waters show high levels of heavy metals which have a toxic effect on microorganisms, plants, animals, and humans. However, previous studies (Goswami *et al*. 2023) affirm how bacteria present in these waters have evolved, through changes in the genome, increasing resistance to these metals; but the most surprising was to find the close relationship present between the mechanism of resistance to metals with the mechanism for antibiotics producing a continuous increase in resistance (Henriquez *et al.*, 2021).

The excessive, uncontrolled, and inappropriate use of antibiotics is another major factor in the rise of AMR. Some studies show that other overcrowded countries such as China produce and use antibiotics in high quantities for the growth of genetically modified animals (Tian *et al*. 2021) where the waste is discharged directly into natural waters which end up highly contaminated.

It has thus been observed that the progressive increase of AMR and the consequent evolution in microorganisms may have a direct effect on human diseases such as *Acanthamoeba* keratitis, where the mechanism of pathogenesis contains several points not yet understood and, therefore, the treatment for it is not yet defined. Furthermore, some studies propose the bacterium-amoeba endosymbiotic hypothesis called ‘Trojan Horse’ (Henriquez *et al.*, 2021) where the bacterium inhibits the phagocytic mechanism of the amoeba to maintain and reproduce itself inside it to go outside and perform a superior infection. Therefore, increasing AMR is described as one of the most important clinical dilemmas nowadays and for this reason, the project study aimed to analyse the AMR for the antibiotics SMZ, ERY, ENR, AZM, LZD and OXA tested in high concentration in the waters of Vashi Creek (Mumbai, India) and to affirm or reject the bacteria-amoeba endosymbiotic hypothesis.

The results of bacterial isolation and AST showed decreasing resistance values over the course of the river for the extracellular bacterial samples. These results contradicted the main hypothesis, as proximity to the reference point was expected to be directly related to AMR. However, this hypothesis did not consider the inflow of seawater into the river, which can mix at low points in the river, decreasing water contamination and thus, the resistance of microorganisms. In contrast, the results of the intracellular samples showed high and stable AMR results along the course of the river, values that reinforce the endosymbiotic hypothesis that allows bacteria to evolve and remain resistant to the antibiotics tested.

Regarding the study of the different antibiotics tested, it was observed that SMZ and OXA were the antibiotics with the highest number of resistant bacterial colonies. However, the project focused only on SMZ, as it had a greater impact on intracellular bacteria and could provide important results for endosymbiotic research. The aim for this was to analyse the minimum inhibitory concentration of SMZ for different bacteria, *Acanthamoeba* and both in coexistence with two main objectives: the determination of the concentration levels of SMZ resistance and the difference in bacterial resistance results present when coexisting with *Acanthamoeba*. These studies were performed for *E. coli* and *Pseudomonas* due to the high concentration of bacteria in the environment and in clinical cases of patients with AK.

From the MIC results for bacteria, it could be observed that SMZ has an inhibitory effect on *E. coli* but not on *Pseudomonas*. These results reinforce the hypothesis of other related studies (Abd El-Ghany 2021) about 100% resistance of *Pseudomonas* towards SMZ. Based on the MIC values for each bacterium, the test was performed for bacteria in coexistence with amoeba in order to observe a positive endosymbiotic effect and thus an increase in bacterial resistance. The results for *E. coli* showed a non-significant resistance capacity in coexistence with *Acanthamoeba*, while *Ps. putida* showed a significant difference in mean SMZ resistance concentrations. Finally, the most prominent results were for *Ps. aeruginosa*, which showed a significant difference in resistance at high SMZ concentrations.

Finally, the study focused on the clinical part where it was demonstrated from PCR for *Acanthamoeba* and bacteria that the presence of these species of microorganisms was present in patients with AK and therefore, the endosymbiotic and evolutionary effects that the bacteria experience have an impact on the disease by increasing their infection. Thus, it is proposed as another element to be considered when investigating a new effective treatment for the disease.

In conclusion, this study has confirmed the endosymbiotic effect between bacterial species and *Acanthamoeba* causing an evolutionary effect on them that has repercussions on the increase of AMR.

6. ACKNOWLEDGEMENTS

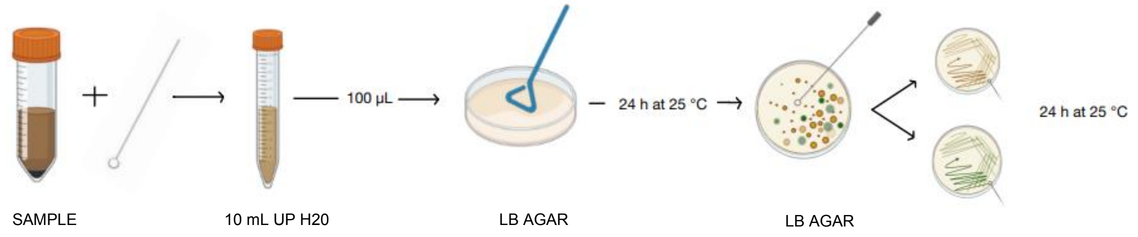
First, I would like to thank the whole A.M.E.G. group at the University of the West of Scotland for hosting me in my internship. I would like to thank Dr Fiona Henriquez and Dr Ronnie Mooney for guiding me all the way and teaching me all the knowledge about microbiology and *Acanthamoeba* keratitis disease. I would also like to thank Dr Erin Corbett and Elisa Giammarini for helping and supporting me every day in the labs and, Richard Childs Hunt for providing the clinical samples for my research.

On the other hand, I would also like to thank my academic tutor Dra. Sandra Guaita Esteruelas for her patience and help throughout the whole process.

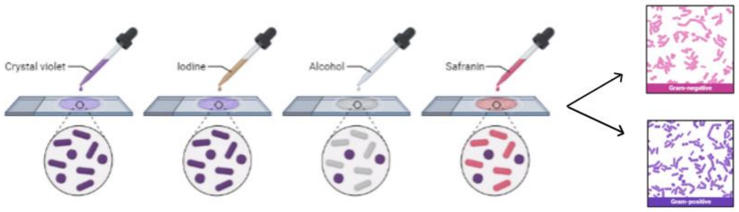
7. ANNEX

A. EXTRACELLULAR ISOLATION METHODOLOGY

1. BACTERIAL ISOLATION AND GROWTH

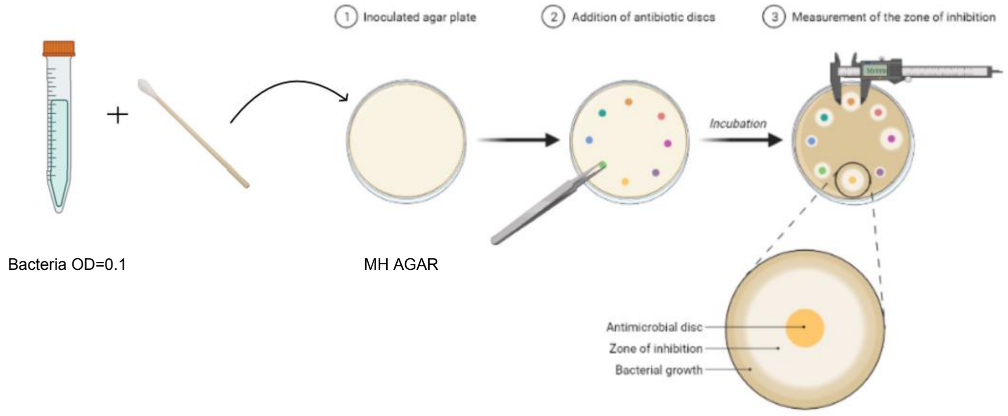


2. GRAM STAINING



GRAM-NEGATIVE	GRAM-POSITIVE
Sulfamethazine (SMZ)	Linezoid (LZD)
Erythromycin (ERY)	Oxacillin (OXA)
Enrofloxacin (ENR)	
Azithromycin (AZM)	

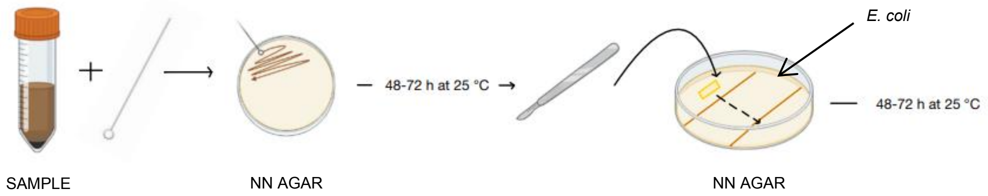
3. ANTIBIOTIC SUSCEPTIBILITY TEST



Created by Biorender

B. INTRACELLULAR ISOLATION METHODOLOGY

1. AMOEBA EXPANSION AND ISOLATION



2. AMOEBA FRAGMENTATION AND BACTERIAL GROWTH

Created by Biorender.

C. EXTRACELLULAR AST RESULTS

S/S 7/10

COLONY	SMZ	ERY	ENR	AZM	DMSO	LZD	OXA
1	36.7	24.7	31	27	0		
2	27	24	37.3	25.3	0		
3	21	24.7	37.7	26.3	0		
4	22.7	30.3	30.7	24.3	0	35	0
5	38	33.3	35.3	29.7	0		
6	28	29.3	44	29.3	0		

S/M 7/10

COLONY	SMZ	ERY	ENR	AZM	DMSO	LZD	OXA
1	38.3	35	33.3	28.7	0		
2	29.7	39.7	37.7	34	0	42	22.7
3	34	35	38.7	36.3	0		
4	30.3	36.7	35	30.3	0		

S/R 7/10

COLONY	SMZ	ERY	ENR	AZM	DMSO	LZD	OXA
1	32.3	28.7	45.7	32	0	24.7	13
2	45.3	39	38	34.3	0		
3	23	33	31.7	26.3	0	37.3	20.3
4	32.3	29	44	29	10.3		
5	37.7	29.7	47	32.3	10.3		

S/1U 7/10

COLONY	SMZ	ERY	ENR	AZM	DMSO	LZD	OXA
1	47.7	45.3	49	39	0	46.3	39
2	29.3	36	29.7	26.7	0	38.7	0
3	37	39	42	29.7	0	42	22.7
4	30.3	20.7	26.3	20.7	0	37.7	0

S/2U 7/10

COLONY	SMZ	ERY	ENR	AZM	DMSO	LZD	OXA
1	26.7	27.7	42	30.3	0		
2	18.7	27.7	34.7	37.3	0		
3	32.7	36.3	36.7	28	0	40.7	0
4	25	11.7	39.7	39	0		

S/1D 7/10

COLONY	SMZ	ERY	ENR	AZM	DMSO	LZD	OXA
1	30	30.3	43.7	35.3	0		
2	23.7	31.3	36.3	35	0		
3	34.7	31.3	50.7	37.3	10		
4	28	30.7	45.7	36	0		
5	35.3	38.3	36.7	30	0	40.3	0
6	39	43.7	39.7	28.3	0	47.3	0
7	28	31.3	42	36	0		

S/2D 7/10

COLONY	SMZ	ERY	ENR	AZM	DMSO	LZD	OXA
1	29.3	31.7	45	34.3	0		
2	31	36.7	39.7	33.3	0		
3	29.7	28	44.7	32.3	0		
4	28.3	28.7	43	33.3	0		
5	30.3	34.7	42.7	38.7	0		
6	48.7	39.7	40.7	34	0		

D. INTRACELLULAR AST RESULTS

S/M 7/10

COLONY	SMZ	ERY	ENR	AZM	DMSO	LZD	OXA
1	38.7	21	38.3	19.7	0		
2	0	21	39.3	20	0		
3	0	23	41	20.7	0		
4	31	21.3	42.3	20.7	0		

S/R 7/10

COLONY	SMZ	ERY	ENR	AZM	DMSO	LZD	OXA
1	0	23	40.3	22	0		
2	39.7	26.7	34.3	20.7	0	39.3	10.7
3	37	25.3	40.3	23.3	0		
4	39.3	20.7	43.7	22.7	0	27.7	0

S/1U 7/10

COLONY	SMZ	ERY	ENR	AZM	DMSO	LZD	OXA
1	30	22.3	40.3	22	0		
2	30	20.7	39.3	20	0		
3	0	20	39	18	0		
4	0	20.3	37	25.7	0		

S/2U 7/10

COLONY	SMZ	ERY	ENR	AZM	DMSO	LZD	OXA
1	35.3	24.3	42.3	22.7	0		
2	0	22.7	41.3	23	0		
3	32.7	21	38.7	0.7	0		
4	0	20.7	40	21.3	0		

S/2D 7/10

COLONY	SMZ	ERY	ENR	AZM	DMSO	LZD	OXA
1	32.3	21.7	42	19.3	0		
2	0	21	39.7	19	0		
3	0	21.7	40.3	21.3	0		
4	34.7	24.7	42.3	23	0		

E. BREAKPOINTS EUCAST, CLSI AND BSAC

ANTIBIOTIC	EUCAST Enterobacterales	EUCAST Staphylococcus	EUCAST Enterococcus	EUCAST Streptococcus A B C G	EUCAST Corynebacterium
Sulfamethazine	Resistant <11 mm	Resistant <14 mm	R esistant <23 mm	Resistant <15 mm	
	Susceptible>= 14mm	Susceptible>= 17mm	Susceptible>= 23 mm	Susceptible>= 18 mm	
Erythromycin		Resistant <21 mm		Resistant <22mm	
		Susceptible>= 21mm		Susceptible>= 22 mm	
Azithromycin	Resistant <12 mm	Resistant <21 mm		Resistant <21 mm (a)	
	Susceotible>= 12mm	Susceptible>= 21mm		Susceptible>= 21mm	
Enrofloxacin					
Oxacillin		Resistant <20 mm		Resistant <20 mm	
		Susceptible >= 20mm		Susceptible >= 20mm	
Linezolid		Resistant <21 mm	Resistant <20 mm	Resistant <19 mm	Resistant <25 mm
		Susceptible >= 21mm	Susceptible >= 20mm	Susceptible >= 19mm	

ANTIBIOTIC	EUCAST Vibrio	EUCAST Bacillus	CLSI Enterobacterales	CLSI Enterobacterales	CLSI Enterococcus	Veterinary pathogens
Sulfamethazine			Resistant <12 mm	Resistant <= 12 mm		
			Susceptible >= 17	Susceptible >= 17mm		
Erythromycin	Resistant <12 mm	Resistant <24 mm		Resistant <= 13mm	Resistant <= 13mm	
				Susceptible >= 23mm	Susceptible >= 23mm	
Azithromycin	Resistant <16 mm		Resistant <12 mm	Resistant <=13 mm		
				Susceptible >= 18mm		
Enrofloxacin						Resistant <= 16
						Susceptible >= 21-23
Oxacillin				Resistant <=17 mm		
				Susceptible >=18mm		
Linezolid		Resistant <22 mm		Resistant <=20 mm	Resistant <=20 mm	
				Susceptible >=21 mm	Susceptible <= 23mm	

a	Inferred from erythro

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