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ORIGINAL ARTICLE

A one health approach versus *Acanthamoeba castellanii*, a potential host for Morganella morganii

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Abstract

Acanthamoeba castellanii, known as the "Trojan horse of the microbial world," is known to host a variety of microorganisms including viruses, yeasts, protists, and bacteria. *Acanthamoeba* can act as a vector and may aid in the transmission of various bacterial pathogens to potential hosts and are found in a variety of places, thus impacting the health of humans, animals, and the environment. These are interconnected in a system known as "one health." With the global threat of antibiotic resistance, bacteria may avoid harsh conditions, antibiotics, and disinfectants by sheltering within *Acanthamoeba*. In this study, *Acanthamoeba castellanii* interaction with *Morganella morganii*, a Gram-negative bacterium was studied. *Escherichia coli* K1 interaction with *Acanthamoeba* was carried out as a control. Association, invasion, and survival assays were accomplished. *Morganella morganii* was found to associate, invade, and survive within *Acanthamoeba castellanii*. Additionally, *Escherichia coli* K1 was also found to associate, invade, and survive within the *Acanthamoeba* at a higher number in comparison to *Morganella morganii*. For the first time, we have shown that *Morganella morganii* interact, invade, and survive within *Acanthamoeba castellanii*, suggesting that *Acanthamoeba* may be a potential vector in the transmission of *Morganella morganii* to susceptible hosts. Taking a one health approach to tackle and develop disinfectants to target *Acanthamoeba* is warranted, as the amoebae may be hosting various microbes such as multiple drug-resistant bacteria and even viruses such as the novel coronavirus.

Keywords *Acanthamoeba* · *Morganella morganii* · Interaction · Survival; Trojan horse; One health

Introduction

Acanthamoeba castellanii is a free-living amoeba that is widely distributed in the environment (Anwar et al. 2019; Mungroo et al. 2021; Scheid et al. 2008; Guimaraes et al. 2016). This free-living amoeba exists in two stages, an active trophozoite stage and a dormant cyst stage (Anwar et al. 2019; Hendiger et al. 2020; Damhorst et al. 2022; Rayamajhee et al. 2021). Over the years, *Acanthamoeba* has been found to interact with various microorganisms such as viruses, fungi, and bacteria (Mungroo et al. 2021; Guimaraes et al. 2016; Rayamajhee et al. 2021). In fact, *Acanthamoeba* is often referred to as the "Trojan Horse of the microbial world," due to its ability to serve as a reservoir for various microorganisms (Yousuf et al. 2013; Mungroo et al. 2021; Guimaraes et al. 2016).

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As it is known, the environment plays a major role in the overall health of humans; one is connected to the other, hence the term "one health" (Solis and Nunn 2021). According to the one health system, the health of humans, animals, and ecosystems is all interconnected (Gruetzmacher et al. 2021). In this regard, various studies have been conducted to understand the role *Acanthamoeba* plays in the survival of various pathogenic bacteria (Yousuf et al. 2013; Mungroo et al. 2021). This is crucial, as bacteria may survive and reproduce within the amoeba, while resisting harsh conditions outside (Mungroo et al. 2021). For instance, in July of the year 1976, 29 people died and 182 were infected with a mysterious illness after attending the 58th State convention of the American Legion Department of Pennsylvania (Siddiqui and Khan 2012). At the time, the disease was not known and was labeled as the Legionnaires' disease. Later in December of 1976, the etiology of the disease was determined to be caused by *Legionella pneumophila*. However, how the bacteria were able to survive in the environment was unknown; it was the microbiologist Tim Rowbotham who viewed the interactions between *Acanthamoeba* and *L. pneumophila*. Hence, he hypothesized that *Acanthamoeba* were able to host *L. pneumophila*, and the mode

of infection was through the inhalation of amoeba infected with *L. pneumophila* (Siddiqui and Khan 2012; Rowbotham 1980). With this observation, the concept of parasite-parasite interactions emerged. Thus, it is important to understand the different interactions two pathogens may have with each other, in order to further understand the emergence of diseases, and understand which individuals may be susceptible. For this reason, in this study, the interactions between the bacterium *Morganella morganii* and *Acanthamoeba castellanii* are studied.

Morganella morganii is a Gram-negative bacterium belonging to the Enterobacteriaceae family (Liu et al. 2016). The growing resistance to drugs makes this bacterium of interest, as it raises the question of how this bacterium is surviving and perhaps even propagating (Liu et al. 2016). Antimicrobial resistance has turned into a global problem as it is responsible for almost 0.7 million deaths (Akbar et al. 2020). Furthermore, to complicate matters, certain species have grown resistance to the various classes of antibiotics; hence, they are termed superbugs (Davies and Davies 2010; Khan and Siddiqui 2014). Although *Morganella morganii* is still not considered a MDR bacteria, if current treatments are not further developed, it may turn into one. This bacterium is responsible for various infections such as urinary tract infections, intra-abdominal infections, and post-operative wound infections (Liu et al. 2016; Dessie et al. 2020). By producing various virulence factors such as urease, lipopolysaccharides, and hemolysins, this bacterium contributes to infections (Liu et al. 2016). Furthermore, it has been found to cause cellulitis, sepsis, abscess, and purple urine bag syndrome (Liu et al. 2016; Seija et al. 2015).

Herein, the interaction between *Morganella morganii* and *Acanthamoeba castellanii* was studied through the conduction of association, invasion, and intracellular survival assays. Further understanding of these interactions and the impact on the one health system are warranted in prospective studies.

Materials and methods

Acanthamoeba culturing

Clinical isolates of *Acanthamoeba castellanii* of the T4 genotype (ATCC 50,492) were cultured in 10 mL of peptone yeast glucose (PYG) growth medium (Anwar, et al. 2019; Baig, et al., 2020). The growth medium was composed of 0.75% proteose peptone, 0.75% yeast extract, and 1.5% glucose. Furthermore, the amoeba was grown in a tissue culture flask which is maintained at 30 ºC; within 48, the flask reaches almost full confluency (Yousuf, et al., 2013).

Bacterial cultures

Escherichia coli K1 and *Morganella morganii* were used in this study as described previously (Soopramanien et al. 2021); Akbar et al. 2021). Prior to the experiment, bacteria were cultured overnight in Luria Bertani (LB) and incubated at 37 ºC (Akbar, et al. 2020; Yousuf, et al. 2013).

Association assays

As mentioned earlier, *Acanthamoeba* was maintained in tissue culture flasks containing the growth medium PYG (Yousuf, et al., 2013). Once confluency was attained, the flask was placed on ice for 20 min to detach the amoeba. Next, the media within the flask was centrifuged for 5 min at a speed of 3000 g to pellet the amoeba. The supernatant was then discarded, and the pellet was resuspended in approximately 2 mL of Roswell Park Memorial Institute (RPMI) medium. The amoeba was then counted using a hemocytometer; the count of amoeba was set to 5 × 105.

Furthermore, the optical density (OD) of the two bacteria used in this study was adjusted. The OD of *Morganella morganii* was adjusted to 0.33 at 595 nm, while the OD for *Escherichia coli* K1 was adjusted to 0.22 at 595 nm. With the following adjustments, there is approximately 108 colony forming units of bacteria. Bacteria and *Acanthamoeba* were incubated together at 30 ºC for 1 h in 24-well plates. Once the incubation period was completed, the amoeba and bacteria were transferred to Eppendorf tubes and resuspended at 2000 g for 5 min. The supernatant was discarded, and the pellet was washed with 500 µL of phosphate-buffered saline (PBS) and vortexed by a brief pulse. This was repeated twice; at the final wash, the discarded supernatant is plated on nutrient agar plates to determine the presence of bacteria (Yousuf, et al. 2013; Siddiqui, et al. 2017). Next, using the hemocytometer, the amoeba was counted. Finally, through the addition of 0.01% sodium dodecyl sulfate (SDS) to the amoeba for 10 min at room temperature, the amoeba was lysed. The bacteria within the amoeba (lysates containing bacteria) were serially diluted and plated onto nutrient agar plates. The following day, the colonies were enumerated, and the percentage of bacterial association was calculated. The ratio of bacteria associated with *A. castellanii* was determined as follows:

Bacteria: *A. castellanii* ratio = recovered bacterial colony forming units / number of *A. castellanii*.

Additionally, the percentage of bacteria associated with *A. castellanii* was determined as follows:

$$\%\,Bacterial colony forming units associated with A.castellanii = recovered bacterial colony forming units / total bacterial colony forming units \times 100\%$$

Invasion assays

By doing invasion assays, the ability of bacteria to invade *Acanthamoeba* is detected (Yousuf, et al., 2013). As described previously for the association assays, the amoeba was grown to complete confluency in tissue culture flasks, and the OD of the bacteria was adjusted. Both the amoeba and bacteria were incubated together

for 1 h and washed with PBS. Once the incubation period is over, 100 µg/mL of gentamicin was added to kill the extracellular bacteria. The bacteria along with the gentamicin added were incubated for 45 min at 30 ºC. When the incubation period was completed, the amoeba was washed with PBS. Furthermore, the PBS was plated onto nutrient agar plates to guarantee that the extracellular bacteria is killed. The amoeba was then counted using a hemocytometer. Furthermore, to determine whether the bacteria were indeed able to invade the amoeba and withstand the effect of gentamicin, the amoeba was lysed using 0.01% SDS. The lysates were then serially diluted and plated onto nutrient agar plates to determine the number of bacteria within the amoeba. To calculate the percentage of bacterial invasion, the following formula was used:

$$\%\ Bacterial\ colony\ forming\ units\ invading\ and\ /or\ taken\ up\ by\ A.\ castellanii = recovered\ bacterial\ colony\ forming\ units\ /total\ bacterial\ colony\ forming\ units \times 100\%$$

Furthermore, the ratio of bacteria to amoeba was calculated using the following formula:

Bacterial colony forming unit: *Acanthamoeba castellanii* ratio = recovered bacterial colony forming unit / number of *Acanthamoeba castellanii* (Siddiqui et al. 2017).

Intracellular survival assays

Intracellular survival assays assess the number of intracellular bacteria within *Acanthamoeba* (Yousuf et al. 2013). Concisely, the amoeba along with the bacteria were incubated for 1 h. After the incubation period was over, 100 µg/mL of gentamicin was added to the samples, and they were incubated for 45 min, as was done for the invasion assay. After the 45-min incubation period, the samples were washed with PBS and incubated for 24 h at 30 ºC. The following day, the amoeba and bacterial colony forming units were counted as described earlier. To determine the percentage of bacteria surviving intracellularly, the following calculation was made: recovered bacterial colony forming units/total bacterial colony forming units × 100 = % intracellular bacterial colony forming units. Furthermore, the ratio of bacteria to amoeba was calculated using the formula: recovered bacterial colony forming units/number of *Acanthamoeba* = bacterial colony forming units: *Acanthamoeba* ratio (Siddiqui et al. 2017).

Results

Morganella morganii displayed association with *Acanthamoeba castellanii*

Association assays were conducted through the incubation of *M. morganii* and *E. coli* K1 with *A. castellanii*. To remove the non-associated bacteria, the amoeba was washed by PBS for three times. During the final wash, the supernatant was plated on nutrient agar plates; however, no bacteria was found. Next, the amoeba was lysed using 0.01% SDS, and the lysates were then plated on nutrient agar plates. The following day, it was revealed that both *M. morganii* and *E. coli* K1 can associate with the *Acanthamoeba*. However, *Morganella morganii* is shown to have reduced association compared to *E. coli* K1. As

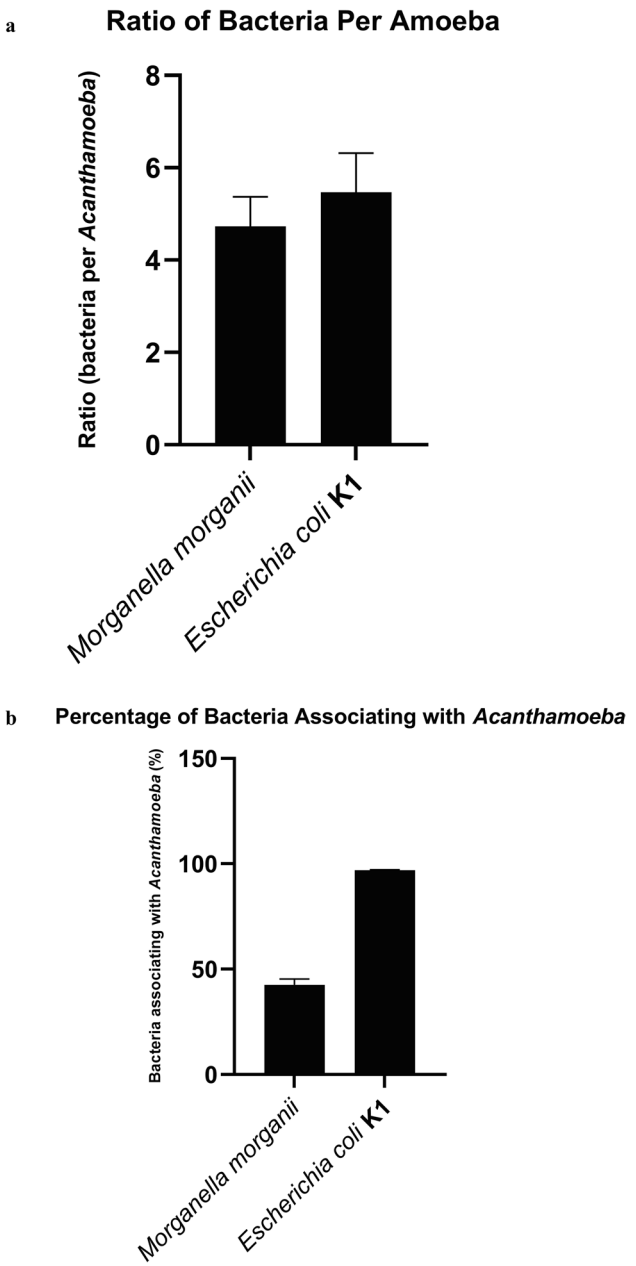

Fig. 1 *Morganella morganii* displayed association with *Acanthamoeba castellanii.* To determine bacterial association, association assays were performed as previously described in the "Methods" section. **A** The ratio of bacteria per amoeba is represented, whereas **B** the percentage of bacteria recovered from the original inoculum is represent. The results are presented as the mean ± standard error of several independent experiments performed in duplicate

Table 1 The percentage of recovered bacteria from *A. castellanii* was determined. The percentage of intracellular bacteria recovered from the association, invasion, and survival assays was determined using the following formula: recovered bacterial colony forming units / total bacterial colony forming unit × 100 = % intracellular bacterial colony forming units

	% recovered bacteria associating from *A. castellanii*	% recovered bacteria invading *A. castellanii*	% recovered bacteria surviving in *A. castellanii*
E. coli K1 and *A. castellanii*	96.9% ± 0.35	1.0% ± 0.05	44.2% ± 2.7
M. morganii and *A. castellanii*	42.6% ± 2.69	0.2% ± 0.08	88.1% ± 5.7

indicated in Fig. 1A, there are five *E. coli* K1 per *Acanthamoeba* (5.41 ± 0.8, bacteria/amoeba ratio). Whereas there is only 3 *M. morganii* per *Acanthamoeba* (3.32 ± 0.03, bacteria/amoeba ratio: Fig. 1A). Additionally, *E. coli* K1 demonstrated higher recovery compared to *M. morganii* (96.9 ± 0.35, 42.6% ± 0.2.69 respectively: Fig. 1B). Tables 1 and 2 depict the % of recovered bacteria from *Acanthamoeba* as well as the ratio of amoeba to bacteria. To assure SDS has no effect on bacterial viability, various concentrations of SDS were tested against bacteria alone and incubated (data not shown).

Morganella morganii displayed invasion of *Acanthamoeba castellanii*

Furthermore, to determine if the intracellular bacteria is capable to invade the amoeba, invasion assays were performed. *M. morganii* was capable of invading and surviving within the *Acanthamoeba*; however, at a lower percentage when compared to *E. coli* K1. As indicated in Fig. 2A, there are seven *E. coli* K1 per ten *Acanthamoeba* (0.68 ± 0.02, bacteria/amoeba ratio: Fig. 2A). Whereas there is one *M. morganii* per ten *Acanthamoeba* (0.13 ± 0.04, bacteria/amoeba ratio: Fig. 2A). Additionally, *E. coli* K1 demonstrated higher recovery compared to *M. morganii* (0.96 ± 0.05, 0.2% ± 0.08 respectively: Fig. 2B).

Morganella morganii displayed intracellular survival within *Acanthamoeba castellanii*

To understand the long-term survival of *M. morganii* and *E. coli* K1 intracellularly within *Acanthamoeba*, intracellular survival assays were performed. Based on the results obtained, it is found that both bacteria can survive within the amoeba. However, *Morganella morganii* is shown to have reduced survival when compared to *E. coli* K1. As indicated in Fig. 3A, there are eight *E. coli* K1 per ten *Acanthamoeba* (0.83 ± 0.07, bacteria/amoeba ratio) whereas there are four *M. morganii* per ten *Acanthamoeba* (0.37 ± 0.004, bacteria/amoeba ratio: Fig. 3A). However, there was greater number of *Morganella morganii* recovered compared to *E. coli* K1 (88% ± 5.7, 44% ± 2.7; respectively: Fig. 3B). Representative images of interactions between *Acanthamoeba castellanii*, *Escherichia coli* K1, and *Morganella morganii* are detailed in Fig. 4.

Discussion

Previously, scientists focused on single-pathogen interactions, and interactions of the host with the surrounding microbial communities and the various species within it were not well considered (Henriquez et al. 2021). It is evident that the eukaryotic unicellular parasite *Acanthamoeba castellanii* is known as the "trojan horse of the microbial world" (Yousuf et al. 2013; Mungroo et al. 2021; Guimaraes et al. 2016; Rayamajhee et al. 2021). Moreover, since the discovery of penicillin by Fleming, the emergence of antimicrobial resistance was observed and attributed to antibiotic resistance genes (Hasan et al. 2022). Following intensive research on pathogens of clinical interest, the concept of the "antibiotic resistome" was first introduced by Gerry Wright in 2006 when he found resistance determinants within soil (Kim and Cha 2021). Further exacerbating the situation is the interactions between bacteria and *Acanthamoeba*, as they may contribute to growing bacterial drug resistance (Mungroo et al. 2021). Moreover, as bacteria may multiply

Table 2 The ratio of recovered bacteria to *A. castellanii* was determined. The ratio of intracellular bacteria within one *A. castellanii.* This was achieved using the following formula: recovered from the association, invasion, and survival assays determined using the following formula: recovered bacterial colony forming units / number of *Acanthamoeba* = bacterial colony forming units: *Acanthamoeba* ratio

	Ratio of associating bacteria per *A. castellanii*	Ratio of invading bacteria per *A. castellanii*	Ratio of surviving bacteria per *A. castellanii*
E. coli K1 and *A. castellanii*	5 ± 0.85	0.68 ± 0.02	0.83 ± 0.07
M. morganii and *A. castellanii*	3 ± 0.04	0.13 ± 0.04	0.37 ± 0.004

within *Acanthamoeba*, they may grow more resistant to drugs, due to the transfer of genetic information, sharing and exchanging drug-resistant genes intracellularly (Mungroo et al. 2021). Additionally, microorganisms found within the amoeba are found to contain a genome substantially larger to that of their relatives, making *Acanthamoeba* a gene melting pot (Mungroo et al. 2021). Given the recent pandemic due to severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2)-induced coronavirus disease-2019, or COVID-19, it is important to understand how these infectious agents are able to survive in external environments. Due to their ubiquitous presence in the environment and their status as the trojan horse of the microbial world, amoebae might also play

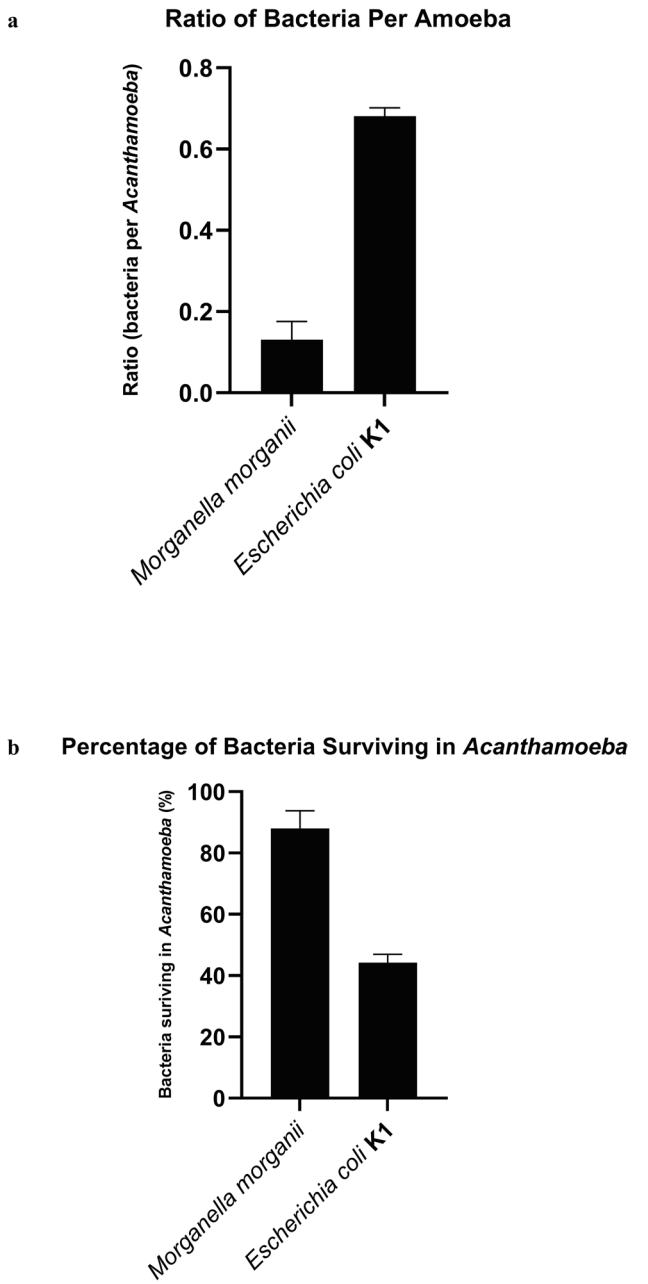

Fig. 2 *Morganella morganii* displayed invasion of *Acanthamoeba castellanii.* To determine bacterial invasion, invasion assays were performed as previously described in the "Methods" section. **A** The ratio of bacteria per amoeba is represent, whereas **B** the percentage of bacteria recovered from the original inoculum is represent. The results are presented as the mean ± standard error of several independent experiments performed in duplicate

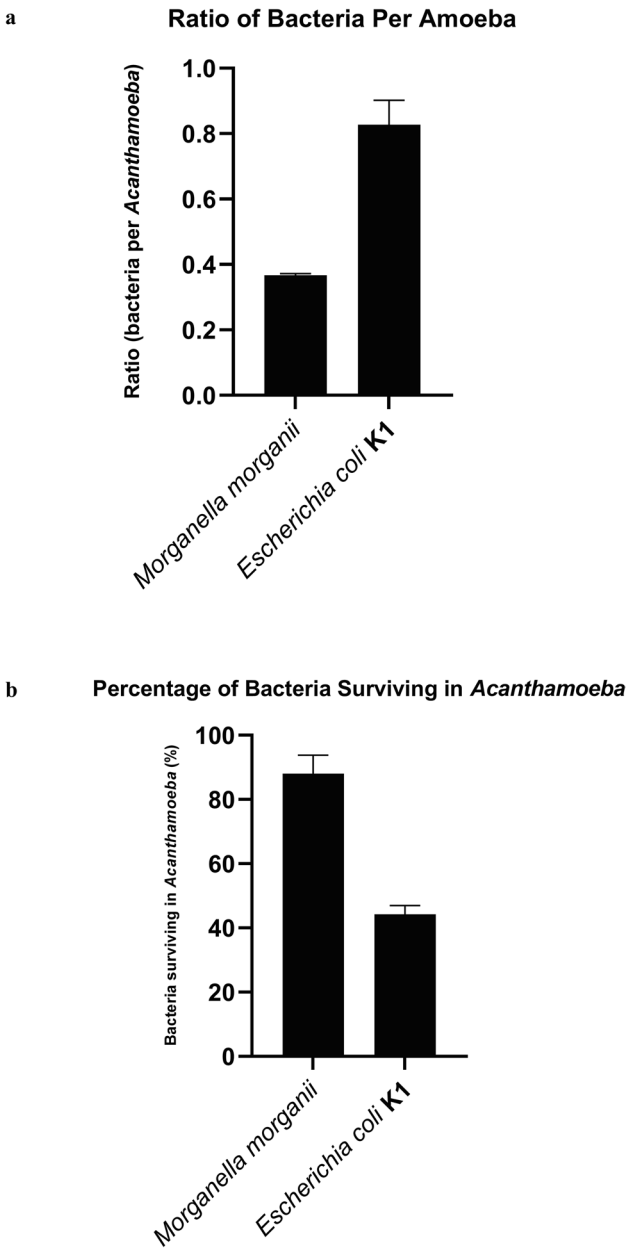

Fig. 3 *Morganella morganii* displayed intracellular survival within *Acanthamoeba castellanii.* To determine bacterial intracellular survival, survival assays were performed as previously described in the "Methods" section. **A** The ratio of bacteria per amoeba is represent, whereas **B** the percentage of bacteria recovered from the original inoculum is represent. The results are presented as the mean ± standard error of several independent experiments conducted in duplicate

a part in providing the novel coronavirus a means to susceptible hosts and possible transmission; hence, it is imperative to study their interaction with microorganisms such as the novel coronavirus as well as other pathogens or microorganisms of interest (Siddiqui and Khan 2020).

In this study, for the first time, the interaction of *Morganella morganii* with *Acanthamoeba* was investigated. In addition, the interaction between *Acanthamoeba castellanii* and *Escherichia coli* K1 was included as a control. It was demonstrated that *M. morganii* are able to associate, invade, and survive intracellularly within amoeba, withstanding the effects of gentamicin. *Morganella morganii* is a bacterium responsible for infections such as post-operative wound infections, urinary tract infections, cellulitis, sepsis, and various others (Liu et al. 2016; Seija et al. 2015). Our results revealed that both *Escherichia coli* K1 and *Morganella morganii* are capable of associating, invading, and surviving within the *Acanthamoeba*, however at different rates. *Morganella morganii* was found to associate, invade, and survive in lesser number compared to *Escherichia coli* K1. This might be due to greater persistence of *E. coli* in nature, as this bacterium is found commonly in nature and is shed by humans and animals (Elsas et al. 2011). Additionally, *E. coli* interacts with the different microbial communities; hence, it may come across *Acanthamoeba* more frequently than *M. morganii* (Elsas et al. 2011). Numerous studies conducted on the interaction between *E. coli* K1 and *Acanthamoeba*

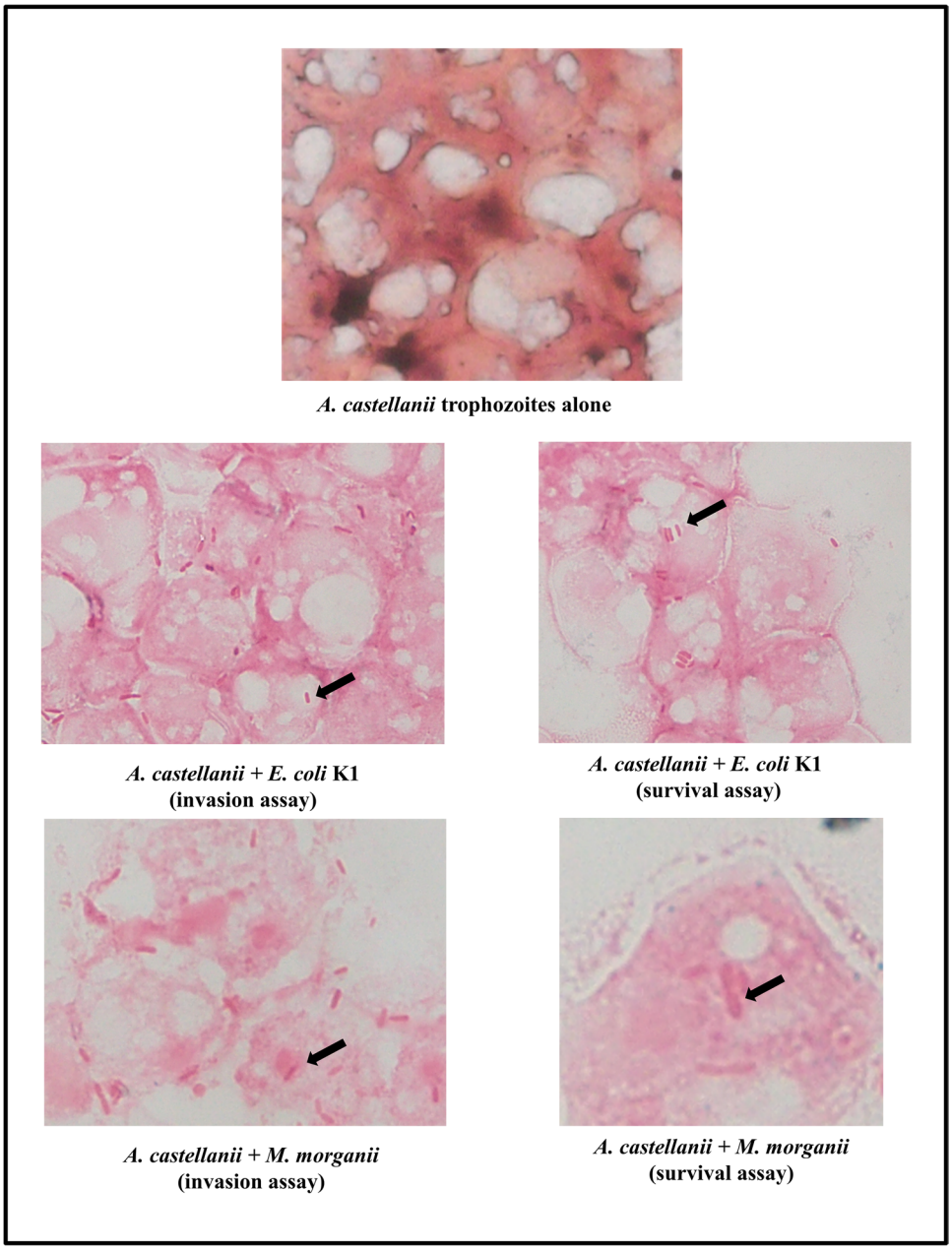

Fig. 4 Representative images of interactions between *Acanthamoeba castellanii*, *Escherichia coli* K1 and *Morganella morganii.* The ability of bacteria to invade and survive within *A. castellanii* was determined through the conduction of invasion and survival assays. The samples were stained using Gram-staining and observed under the microscope. The arrows indicate the bacteria present (×400 for micrograph showing *A. castellanii* trophozoites alone and other micrographs were taken at × 1000)

hypothesized that *E. coli* K1's ability to enter, survive, and replicate within *Acanthamoeba* is due to the resemblance of *Acanthamoeba* to macrophages; they are similar in their cell surface receptors and phagocytic activities (Alsam et al. 2006; Matin and Jung 2011; Jung, et al., 2006)¬¬. This resemblance is crucial for the survival of *E. coli* K1 within the bloodstream, as it impacts the development of meningitis (Alsam et al. 2006). Furthermore, studies have investigated the interaction between *Acanthamoeba polyphaga* and the bacteria: *Enterococcus faecalis*, *Bacillus cereus*, *Listeria monocytogenes*, Enteropathogenic *Escherichia coli*, *Salmonella enterica* serovar Tryphimurium, and methicillin-sensitive *Staphylococcus aureus*; in this study, *L. monocytogenes* and *S. aureus* were found to have significantly higher extracellular numbers when cultured with amoeba compared to when alone (Huws et al. 2008).

Further comprehension of the survival mechanism of *M. morganii* in *Acanthamoeba* is warranted so it may be possible to target and modify various treatments and disinfectants currently utilized against microorganisms (Liu et al. 2016; Seija et al. 2015). This is crucial as both amoebae and the microbes it is hosting, maybe pathogenic; thus, a hyperparasitic relationship is maintained, and targeting the multiple microorganisms rather than just one at a time may be a good strategy (Siddiqui and Khan 2017).

Previously, it has been suggested that further developing disinfectants into targeting the host cell *Acanthamoeba* may aid in the elimination of superbugs (Khan and Siddiqui 2014). For example, chlorine bleach, a cost-friendly disinfectant commonly used, is not effective against *Acanthamoeba* but is effective against several common pathogens. Thus, one suggestion may be to utilize a disinfectant which is also effective against *Acanthamoeba*. Combatting *Acanthamoeba* is crucial as they are capable of remaining viable and retaining their pathogenicity for over 20 years (Siddiqui and Khan 2017). However, to achieve these developments, the molecular mechanism of the bacteria's localization within the *Acanthamoeba* should be determined. Additionally, the mechanisms by which the *Acanthamoeba* takes in bacteria and releases it within the host should also be determined in further studies. Furthermore, whether amoebae are able to host the novel coronavirus needs to be determined, given the omnipresent nature of amoebae as well as the presence of the virus in wastewater (Siddiqui et al. 2020), and disinfectants should be designed accordingly.

Conclusion

For the first time, it has been shown that *Morganella morganii* is capable of associating, invading, and surviving within *Acanthamoeba castellanii*. This is noteworthy, as amoebae can host various pathogens that may spread within our ecosystem contributing to infection in humans and animals, known as "one health," where the health of all three humans, animals, and the environment is interconnected (Solis and Nunn 2021; Gruetzmacher et al. 2020). The comprehension of pathogens or microorganisms that are capable of surviving within *Acanthamoeba* will open the path to develop appropriate disinfectants to target not just bacteria but also the host: *Acanthamoeba* as well. Moreover, comprehension of the molecular basis of these interactions, such as changes in gene expressions or receptors utilized by microbes, will provide insight in how pathogenicity and antibiotic resistance can be modulated, as well as understanding the role of *Acanthamoeba* in health and disease, and encouraging a one health approach.

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Author contribution RS and NAK conceived the study amid discussion with AB. AB and NA performed all experiments under the supervision of RS and NAK. AB and RS prepared the first draft. NAK and RS corrected the manuscript. All the authors approved the final manuscript.

Data availability Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Declarations

Ethics approval and consent to participate This article does not contain any studies with human participants. This article does not contain any studies involving animals.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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