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Interaction of *Escherichia coli* K1 and K5 with *Acanthamoeba castellanii* Trophozoites and Cysts

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Abstract: The existence of symbiotic relationships between *Acanthamoeba* and a variety of bacteria is well-documented. However, the ability of *Acanthamoeba* interacting with host bacterial pathogens has gained particular attention. Here, to understand the interactions of *Escherichia coli* K1 and *E. coli* K5 strains with *Acanthamoeba castellanii* trophozoites and cysts, association assay, invasion assay, survival assay, and the measurement of bacterial numbers from cysts were performed, and nonpathogenic *E. coli* K12 was also applied. The association ratio of *E. coli* K1 with *A. castellanii* was 4.3 cfu per amoeba for 1 hr but *E. coli* K5 with *A. castellanii* was 1 cfu per amoeba for 1 hr. By invasion and survival assays, *E. coli* K5 was recovered less than *E. coli* K1 but still alive inside *A. castellanii*. *E. coli* K1 and K5 survived and multiplied intracellularly in *A. castellanii*. The survival assay was performed under a favourable condition for 22 hr and 43 hr with the encystment of *A. castellanii*. Under the favourable condition for the transformation of trophozoites into cysts, *E. coli* K5 multiplied significantly. Moreover, the pathogenic potential of *E. coli* K1 from *A. castellanii* cysts exhibited no changes as compared with *E. coli* K1 from *A. castellanii* trophozoites. *E. coli* K5 was multiplied in *A. castellanii* trophozoites and survived in *A. castellanii* cysts. Therefore, this study suggests that *E. coli* K5 can use *A. castellanii* as a reservoir host or a vector for the bacterial transmission.

Key words: *Acanthamoeba castellanii*, *Escherichia coli* K1, *Escherichia coli* K5, association, invasion, survival

INTRODUCTION

Acanthamoeba is a free-living protozoan pathogen that has been isolated from diverse environments, including air, soil, tap water, swimming pools, and is known to be one of the most ubiquitous protozoans [1-3]. With the wide environmental distribution, they must effectively interact with a range of microorganisms to ensure their survival. It is not surprising that *Acanthamoeba* have been shown to interact with viruses, bacteria, algae, yeast, and other protists [4-7]. However, it is the ability of *Acanthamoeba* interacting with host bacterial pathogens that has gained most attention. For example, several studies have shown that *Acanthamoeba* act as a reservoir for a variety of bacterial pathogens, including *Escherichia coli* K1 (causative agent of meningitis) [1,8,9], *Legionella pneumophila* (causative agent of Legionnaire's disease) [10], *Coxiella burnetii* (causative agent of Q fever) [11], *Pseudomonas aeruginosa* (causative agent of keratitis) [12], *Vibrio cholerae* (causative agent of cholera) [13], *Helicobacter pylori* (causative agent of gastric ulcers) [14], *Listeria monocytogenes* (causative agent of listeriosis) [15], *E. coli* O157 (causative agent of hemolytic uremic syndrome) [16], and *Mycobacterium avium* (causative agent of respiratory infections) [17] and may act as vectors to transmit these pathogens to susceptible hosts.

The endosymbiotic relationship of bacteria with amoeba may serve (i) to protect bacteria in hostile environments, (ii) the amoebic intracellular environment might assist bacteria to adapt to survival in mammalian phagocytic cells, suggesting that amoeba-bacteria are involved in complex interactions. Further complexity is attributed to the fact that bacteria are a favourable food source for *Acanthamoeba*. What determines amoeba to act as a host for bacteria and at other times to consume bacteria remains incompletely understood. The fate of bacteria inside *Acanthamoeba* may be most likely dependent on the virulence properties of bacteria and/or *Acanthamoeba*. Based on this, it is hypothesized that the ability of bacteria to resist amoebic killing may have led to their evolution to produce human diseases, i.e., evade human immune cells, such as

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phagocytes. Therefore, amoebae may be the missing link to bacteria that are distributed in the environment and those causing human infections.

Most bacteria contain some sort of a polysaccharide layer outside the cell wall or outer membrane. In a general sense, this layer is called a capsule. Capsules are known to protect bacteria from engulfment by predatory phagocytes and from attack by antimicrobial agents. As related with previous studies, invasive *E. coli* K1 interacted with *A. castellanii* trophozoites more than non-invasive *E. coli* K12, which meant more association, invasion, and survival of *E. coli* K1 with *A. castellanii* [1,8]. In particular, a capsule-deletion mutant of *E. coli* K1 (lacking the neuDB genes cluster that is necessary for the production of cytoplasmic precursors to the exopolysaccharide capsule) exhibited significantly reduced association compared with the wild type strain and limited ability for invasion/uptake by and survival inside *Acanthamoeba*. The bacterial capsule would be a very important factor for the interactions with amoeba. However, the importance of a somatic antigen as other virulence factor in *E. coli* is not clearly understood. *E. coli* K5 is a streptomycin-resistant strain of the serotype O7:K1 [18]. *E. coli* K1 and *E. coli* K5 possess different somatic antigens but an identical capsular antigen.

Here, to understand *E. coli*-interactions with amoeba using *E. coli* strains possessing different somatic antigens, we compared and analyzed the *E. coli* K1 and *E. coli* K5 interactions with *A. castellanii* using association, invasion, and intracellular survival assays in *A. castellanii* trophozoites and cysts.

MATERIALS AND METHODS

Culture of *Acanthamoeba* trophozoites and *E. coli*

All chemicals were purchased from Sigma Laboratories (Poole, Dorset, England), unless otherwise stated. A clinical isolate of *A. castellanii* belonging to T4 genotype, isolated from a keratitis patient (American Type Culture Collection, ATCC 50492) was used. *A. castellanii* isolate was grown without shaking in 15 ml of PYG medium [proteose peptone 0.75% (w/v), yeast extract 0.75% (w/v), and glucose 1.5% (w/v)] in T-75 tissue culture flasks at 30℃ as previously described [19]. The media were refreshed 17-20 hr prior to experiments. This resulted in more than 95% amoebae in the trophozoite forms, which were subsequently used in experiments. A laboratory non-invasive *E. coli* strain HB101 (K12) was used as a non-pathogenic bacteria. The *E. coli* K1 strain E44 used in the present study is a rifampicin-resistant mutant of strain RS218 (serotype O18:K1). This strain is a clinical isolate derived from the cerebrospinal fluid (CSF) of a neonate with meningitis. In particular, *E. coli* K5 is a streptomycin-resistant strain of serotype O7:K1 and were cultured with streptomycin (25 μg/ml) at 37℃ overnight before use.

E. coli association assay

To study *E. coli* interactions with live *Acanthamoeba*, an association assay was performed as was done in a previous report [9]. Briefly, *Acanthamoeba* were prepared in 24-well plates. The cells were washed once with PBS and incubated with *E. coli* strains (2×10^6 cfu/well/0.5 ml of PBS). The plates were incubated for 1 hr at room temperature. Following this incubation, amoebae were washed with PBS for 3 times to remove non-adherent bacteria and amoebae counted using a hemacytometer. Finally, amoebae were lysed by adding SDS (0.5% final conc.) to each well for 30 min, and the number of bacteria was enumerated by plating on nutrient agar plates. The percent bacterial association was calculated as follows: recovered *E. coli* (cfu)/total *E. coli* (cfu) × 100 = % *E. coli* associated with *Acanthamoeba*. In addition, the ratio of bacteria to amoebae was calculated as follows: recovered *E. coli* (cfu)/number of *Acanthamoeba* = *E. coli*/*Acanthamoeba* ratio.

E. coli invasion assay

To determine the ability of bacteria to invade or be taken up by *Acanthamoeba*, an invasion assay was performed. Briefly, amoebae were grown to confluency in 24-well plates followed by the addition of 2×10^6 of *E. coli* as described above. After 1 hr incubation, *Acanthamoeba* were washed with PBS for 3 times, followed by an addition of gentamicin (100 μg/ml, final concentration, for 45 min) to kill extracellular bacteria. Finally, amoebae were counted, and the intracellular bacteria were enumerated as described above. The percent bacterial invasion/uptake was calculated as follows; recovered *E. coli* (cfu)/total *E. coli* (cfu) × 100 = % intracellular *E. coli*. In addition, the ratio of bacteria to amoebae was calculated as follows; recovered *E. coli* (cfu)/number of *Acanthamoeba* = *E. coli*/*Acanthamoeba* ratio.

Intracellular survival assay

To determine the long-term effects of *Acanthamoeba* and *E. coli* interactions, an intracellular survival assay was performed. Briefly, amoebae were incubated with *E. coli*, followed by an

addition of gentamicin for 45 min. After this incubation, *Acanthamoeba* were washed for 3 times with PBS and subsequently incubated in 0.5 ml of PBS for 24 hr at 30℃. Finally, amoebae and *E. coli* were enumerated as described above and intracellular bacteria calculated as follows: recovered *E. coli* (cfu)/total *E. coli* (cfu) × 100 = % intracellular *E. coli* after 24 hr in PBS. In addition, the ratio of bacteria to amoebae was calculated as follows: recovered *E. coli* (cfu)/number of *Acanthamoeba* = *E. coli*/*Acanthamoeba* ratio after 24 hr in PBS.

Encystment assay

An encystment assay was performed as previously described [20]. In the invasion assay, 24-well plates were added with gentamicin and then washed with PBS mentioned above. All mixture from a 24-well plate was transferred into a 1.5 ml eppendorf tube. After the tube was centrifuged, the supernatant was removed, and 50 µl PBS was added into the tube. Then, the 50 µl mixture was inoculated on 3% non-nutrient agar, that is, purified agar (Oxoid, Basingstoke, Hampshire, England) plates. The plates were incubated at 30℃ for up to 48 hr. The encystment of *Acanthamoeba* was periodically observed under a light microscope.

Bacterial survival from *Acanthamoeba* cysts induction

After non-nutrient agar surfaces were soaked with 10 ml of PBS by shaking, cysts on the non-nutrient agar plates were gently scraped-off the agar surface using a cell scraper. Cysts were collected by centrifugation at 3,500 rpm for 10 min, washed 3 times with PBS, transferred into a 1.5 ml eppendorf tube with 700 µl of PBS and used in subsequent survival assays. One hundred µl of 700 µl mixture was added into a 24-well plate for a 0 hr survival assay, followed by the addition of SDS (0.5% final concentration) to solubilize any remaining trophozoites as described above. Two 100 µl of 600 µl mixture was added into a 24-well plate containing 900 µl of PYG media for a 43 hr survival assay at 30℃. Two 200 µl of 400 µl mixture was added into a 24-well plate containing PYG and gentamicin (100 µg/ml) for 45 min at 30℃. At the experimental time point, SDS (0.5%) was added to solubilize any remaining trophozoites as described above. Finally, amoebae and *E. coli* were enumerated as described above and intracellular bacteria calculated as follows: recovered *E. coli* (cfu)/total *E. coli* (cfu) × 100 = % intracellular *E. coli* after 22 hr and 43 hr in PYG. In addition, the ratio of bacteria to amoebae was calculated as follows: recovered *E. coli* (cfu)/number of *Acanthamoeba* = *E. coli*/*Acanthamoeba* ratio after 22 hr and 43 hr in PYG.

RESULTS

E. coli K5 exhibited significantly lower association with *A. castellanii* as compared to the invasive *E. coli* K1

To determine the ability of *E. coli* K5 association with *A. castellanii*, the association assay was performed. Our findings revealed that the invasive *E. coli* K5 exhibited significantly lower

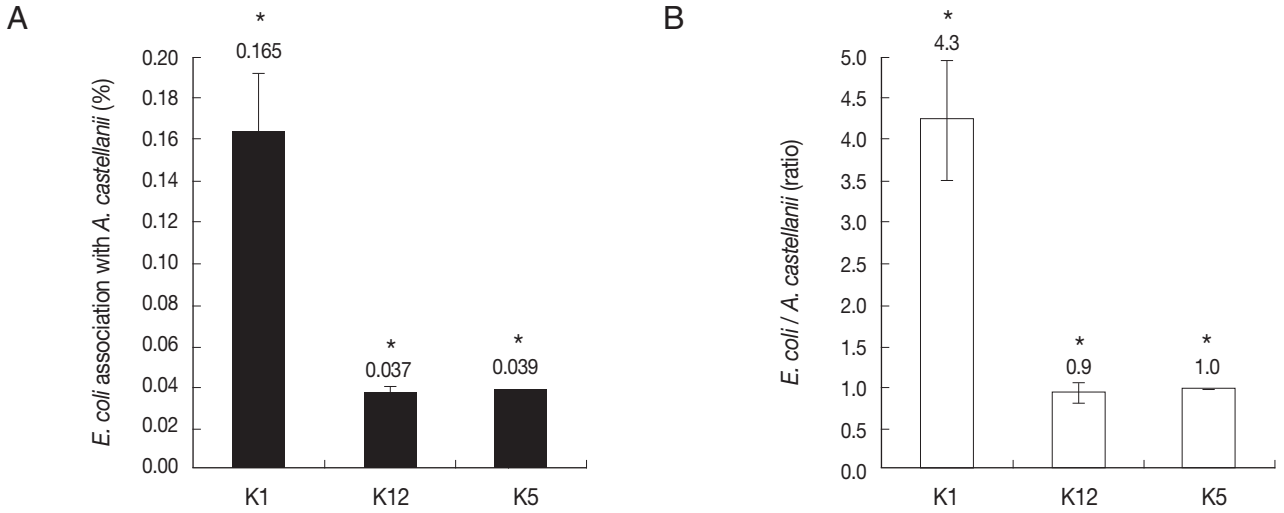

Fig. 1. *E. coli* K5 exhibits significantly lower association with *A. castellanii* as compared to the invasive *E. coli* K1. To determine the ability of *E. coli* to associate with live *A. castellanii*, the association assay was performed. 'A' represents bacterial association with *A. castellanii*, while 'B' represents cfu per amoeba. Results are representative of 3 independent experiments performed in triplicate. Asterisks indicate a significant difference, i.e., $P < 0.05$, using a paired *t*-test, one-tail distribution.

association with *A. castellanii* as compared to the invasive *E. coli* K1 for 1 hr (Fig. 1) ($P<0.05$). Otherwise, the association of *E. coli* K5 was very similar to the non-invasive *E. coli* K12. Next, to determine the ratio of *E. coli* with *A. castellanii*, amoebae were enumerated prior to bacterial counting. Our results revealed that the ratio of *E. coli* K1 with *A. castellanii* was 4.3 cfu per amoeba (0.9 cfu per amoeba for K12) for 1 hr. In particular, the ratio of *E. coli* K5 with *A. castellanii* was 1 cfu per amoeba for 1 hr. *E. coli* K5 exhibited significantly lower association with *A. castellanii* as compared to *E. coli* K1, suggesting that the somatic antigen but not a capsular antigen might be an important factor that interacted with *A. castellanii* trophozoites.

E. coli K5 exhibited significantly decreased invasion into or being uptaken by *A. castellanii* as compared to the invasive *E. coli* K1

Next, to determine the *E. coli* intracellular of amoebae, the invasion assay was performed. We observed a higher recovery (0.008 bacterial colony per amoeba) of *E. coli* K1 intracellular of *A. castellanii* as compared to K12 (no bacterial colony per amoeba) for 1 hr (Fig. 2) ($P<0.05$). Interestingly, *E. coli* K5 was recovered less (0.00011 bacterial colonies per amoeba) than *E. coli* K1 but still alive inside *A. castellanii* for 1 hr ($P<0.05$).

E. coli K5 survived intracellularly in *A. castellanii* while non-invasive *E. coli* K12 were killed

To determine the fate of *E. coli* in long-term interactions with *A. castellanii*, the intracellular survival assay was performed by incubating *E. coli* with *Acanthamoeba* in PBS for 24 hr. With regard to the clinical isolates of *Acanthamoeba*, our findings revealed that once intracellular, *E. coli* K1 remained viable and multiplying, while K12 were killed (Fig. 3) ($P<0.05$). Interestingly, *E. coli* K5 survived and multiplied a little intracellularly in *A. castellanii*. It is suggested that intracellularly invaded *E. coli* K5 multiply in the cytoplasm of *A. castellanii*.

E. coli K5 survived inside the cyst form of *A. castellanii* as compared with *E. coli* K1

After *A. castellanii* was incubated with bacteria for 1 hr and then treated with gentamicin for 45 min, all *Acanthamoeba* trophozoites were inoculated onto non-nutrient agar plate to induce encystment of *A. castellanii*. The encystment was observed periodically up to 43 hr. The groups of *A. castellanii* cysts were grape-shaped as well as tightly linked (Fig. 4). Invasive *E. coli* K1 survived intracellularly and/or extracellularly in *A. castellanii* at 0 hr (0.052 bacterial colony per amoeba; 0.044 cfu per amoeba) (Fig. 5) ($P<0.05$). Otherwise, *E. coli* K12 and *E. coli* K5 didn't survive intracellularly and/or extracellularly in *A. castellanii* at 0 hr. To determine whether a very few number of *E.*

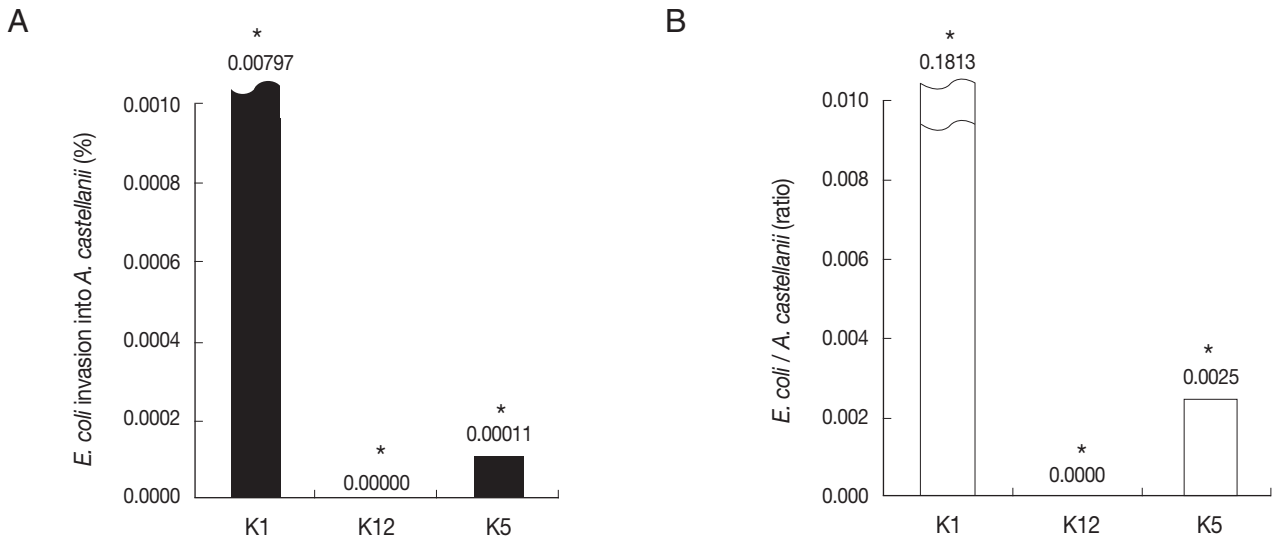

Fig. 2. *E. coli* K5 exhibits significantly lower invasion and/or uptake by *A. castellanii* as compared to the invasive *E. coli* K1. To determine the ability of *E. coli* to invade and/or be taken up by *A. castellanii*, the invasion assay was performed. 'A' represents bacterial association with *A. castellanii*, while 'B' represents cfu per amoeba. Results are representative of 3 independent experiments performed in triplicate. Asterisks indicate a significant difference, i.e., $P<0.05$, using a paired *t*-test, one-tail distribution.

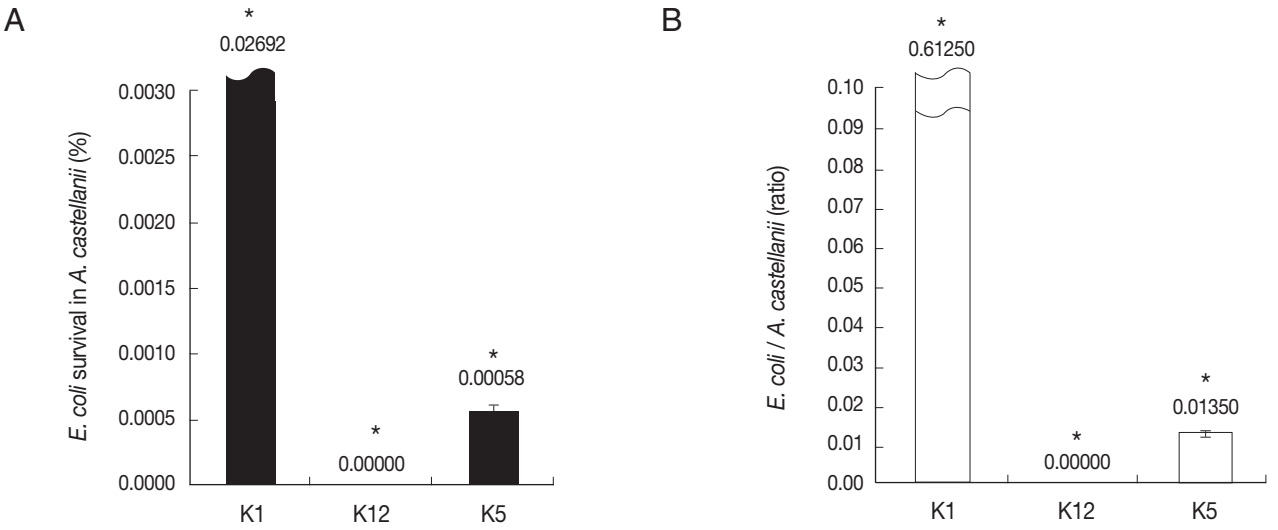

Fig. 3. *E. coli* K5 survives a little intracellularly while K12 are killed under unfavourable conditions. To determine the ability of *E. coli* to survive intracellularly, the survival assay was performed. 'A' represents bacterial association with *A. castellanii*, while 'B' represents cfu per amoeba. Results are representative of 3 independent experiments performed in triplicate. Asterisks indicate a significant difference, i.e., $P<0.05$, using a paired *t*-test, one-tail distribution.

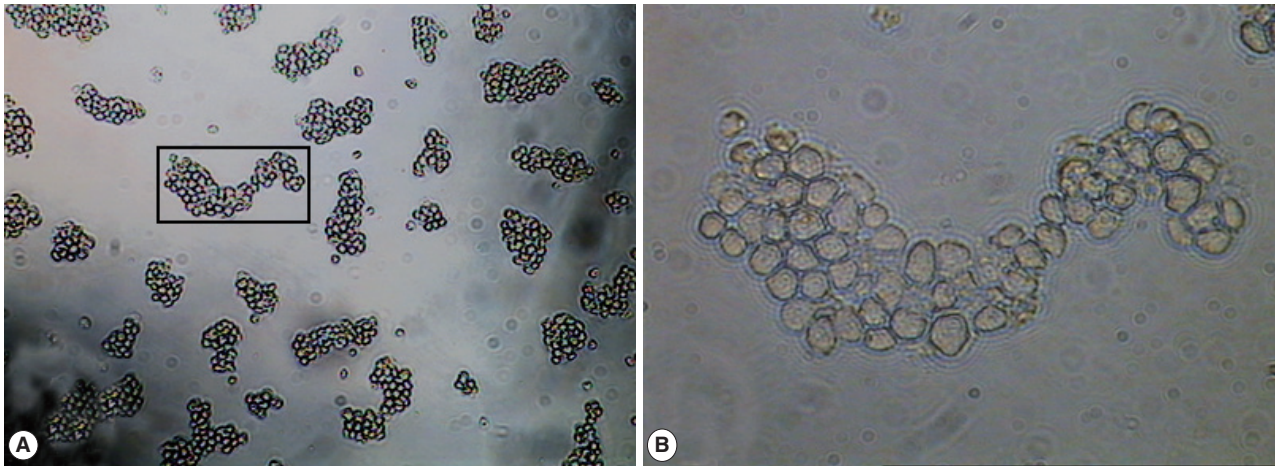

Fig. 4. Encystment of *A. castellanii*. Of the invasion assay, after gentamicin was treated to *A. castellanii* for 45 min, all *A. castellanii* were inoculated onto non-nutrient agar plate to induce encystment of *A. castellanii* up to 43 hr. It was observed that *A. castellanii* cysts formed grape-shaped group linked tightly. 'A' represents *A. castellanii* cysts observed with ×200 magnification power. Inset box was observed with ×400 magnification power under a light microscope in 'B'.

coli K5 may survive intracellularly in *A. castellanii*, cyst forms of *A. castellanii* were incubated under a favourable condition of PYG medium up to 43 hr, which would exhibit transformation of cysts into trophozoites and bacteria might come out of *A. castellanii* trophozoites. Under the favourable condition, no non-invasive *E. coli* K12 were come up but *E. coli* K5 multiplied significantly up to estimated 43 hr. In addition, the number of *A. castellanii* trophozoites was not significantly increased during 22 hr and 43 hr more than 0 hr (data not shown). Very interestingly, *E. coli* K5 multiplied and survived in transformed *A. castellanii* trophozoites under favourable conditions, suggesting that *E. coli* K5 in *A. castellanii* cysts be alive and come out of trophozoites under favourable conditions.

Pathogenic potential of *E. coli* K1 from *A. castellanii* cysts exhibited no changes as compared with *E. coli* K1 from *A. castellanii* trophozoites

To determine whether invasive *E. coli* K1 from *A. castellanii*

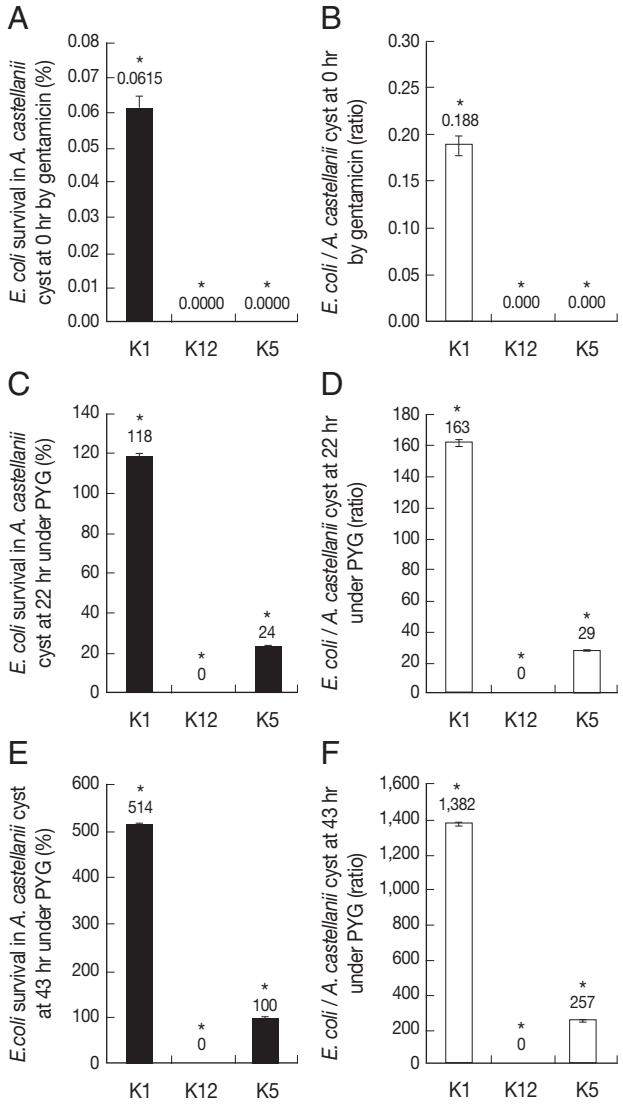

Fig. 5. *E. coli* K5 survived extracelluarly in *A. castellanii* cysts. Encystment of *A. castellanii* was performed as mentioned above. For 22 hr and 43 hr survival assays under favourable conditions of PYG media, the cysts were treated with gentamicin for 45 min to remove extracellular *E. coli*. 'A' and 'B' represent the survival of *E. coli* from *A. castellanii* cysts at 0 hr with treatment of gentamicin. 'C' and 'D' represent the survival of *E. coli* from *A. castellanii* cysts at 22 hr under favourable conditions of PYG media. 'E' and 'F' represent the survival of *E. coli* from *A. castellanii* cysts at 43 hr under favourable conditions of PYG media. A, C, and E represent bacterial association with *A. castellanii*, while B, D, and F represent ratio of bacteria per amoeba. Results are representative of 3 independent experiments performed in triplicate. Asterisks indicate a significant difference, i.e., $P<0.05$, using a paired *t*-test, one-tail distribution.

trophozoites may have different pathogenic potential from invasive *E. coli* K1 from *A. castellanii* cysts, the association, invasion, and survival assays were performed as mentioned above.

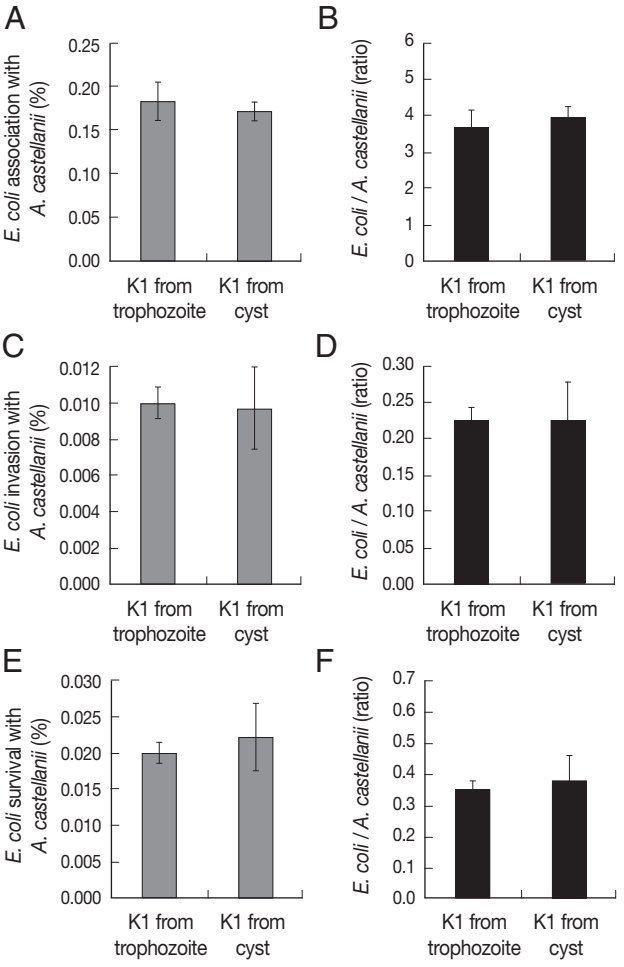

Fig. 6. The pathogenic potential of *E. coli* K1 from *A. castellanii* cysts was not changed as compared with *E. coli* K1 from trophozoites. *E. coli* K1 from *A. castellanii* cysts and trophozoites was continuously assessed by association, invasion, and intracellular survival assays mentioned above. 'A' and 'B' represent association assays. 'B' and 'C' represent invasion assays. 'E' and 'F' represent intracellular survival assays. A, C, and E represent bacterial association with *A. castellanii*, while B, D, and F represent ratio of bacteria per amoeba. Results are representative of 3 independent experiments performed in triplicate. Asterisks indicate a significant difference, i.e., $P<0.05$, using a paired *t*-test, one-tail distribution.

The ability of *E. coli* K1 from *A. castellanii* cysts to interact with *A. castellanii* trophozoites exhibited little changes as compared with *E. coli* K1 from *A. castellanii* trophozoites to interact with *A. castellanii* trophozoites (Fig. 6). These results revealed that although the intracellularly survived *E. coli* K1 from *A. castellanii* cysts multiplied, the pathogenic potential of the *E. coli* K1 might have never changed.

DISCUSSION

The predatory role of *Acanthamoeba* in the control of bacterial populations and their ability to host bacteria and/or to act as a reservoir for bacterial pathogens suggests that these protists play important roles in the environment and contribute to human infections with bacteria. For the latter, a clear evidence is that the environmental bacterium, *L. pneumophila* parasitize *Acanthamoeba* as well as use it as a host or a vehicle to infect the human lung macrophages. The ability of *L. pneumophila* to survive intracellularly in *Acanthamoeba* has been suggested to be a key step in the evolution of this environmental bacterium to produce human infections. This concept is further strengthened with the finding that *Acanthamoeba* resembles human macrophages in many ways, particularly in their phagocytic activity and cell surface receptors [21] and that macrophages and *Acanthamoeba* exhibit parallel mechanisms in their interactions with *L. pneumophila* [22].

Here, to determine the differential interactions of *E. coli* K5 and *E. coli* K1 with *A. castellanii*, we analyzed association with, invasion into, and survival of these bacteria in *A. castellanii*. In previous studys, *E. coli* K1 of other capsular antigen compared with *E. coli* K12 were highly associated, invaded, and intracellularly survived in *A. castellanii* [1,8]. Interestingly, LPS chemical mutant of *E. coli* K1 exhibited less association, invasion, and survival in *A. castellanii* as compared with invasive *E. coli* K1. Beside FimH, both OmpA and LPS were crucial in *E. coli* K1 binding, association, and subsequent survival and replication within *A. castellanii* tested. This finding showed remarkable similarities with human macrophages. For example, recent studies have shown that *E. coli* K1 but not K12 are able to enter, survive, and replicate intracellularly in murine and human macrophages [23]. This property may be crucial for *E. coli* K1 survival in the bloodstream, a primary step in the development of meningitis.

As shown in this study, *E. coli* K5 exhibited significantly low association, invasion, and intracellular survival in *A. castellanii* trophozoites as compared with *E. coli* K1. It survived in *A. castellanii* cysts. Although *A. castellanii* cysts were transformed into trophozoites under PYG media, no *E. coli* K5 survived up to 0 hr which indicated the invasion assay. Thereafter, *E. coli* K5 survived in *A. castellanii* cysts and also recovered in transformed *A. castellanii* trophozoites under favourable conditions. Taken together, they implied that *A. castellanii* trophozoites more active in phagocytosis and movement than cysts would engulf *E. coli* K5. In fact, *E. coli* K1 differs from *E. coli* K5 by the somatic antigen but not capsular antigen. *E. coli* K5 showed less association, invasion, and survival as compared with *E. coli* K1 with *A. castellanii* trophozoites and cysts. More importantly, the pathogenic potential of *E. coli* K1 was not changed as shown in Fig. 6.

Previous studies have shown that the attachment of *L. pneumophila* to the protozoan host, *Hartmanella vermiformis* is mediated by galactose/N-acetylgalactoseamine residues on the surface of bacteria that are recognized by a 170 kDa receptor lectin on the surface of protozoa [24,25]. In contrast, adherence of *L. pneumophila* to macrophages is dependent on heat-labile serum opsonins [26]. However, the mechanisms of subsequent survival and replication of *L. pneumophila* within protozoa and macrophages are remarkably similar [22,27-30]. The fact that *Acanthamoeba* resembles human macrophages in many ways, particularly in their phagocytic activity [9], and that macrophages and *Acanthamoeba* exhibit parallel mechanisms in their interactions with *E. coli*, suggest that *Acanthamoeba* may provide an alternative model to study *E. coli* pathogenesis as well as to understand bacterial immune evasion strategies. Future studies will establish this hypothesis and will precisely determine whether *E. coli* K5 survival and multiplication intracellularly of *Acanthamoeba* affects its virulence.

Overall, these studies suggest that *E. coli* K5 interactions with *A. castellanii* trophozoites are not significantly increased as compared with *E. coli* K1. Otherwise, *E. coli* K5 interactions were very similar with non-invasive *E. coli* K12. However, the interaction of *E. coli* K5 and *A. castellanii* cysts was obviously different from that of *E. coli* K12. Therefore, although the capsular serotypes were identical, these results suggested that the different somatic antigen in *E. coli* K5 would be a very important role in association, invasion, and survival of *E. coli* K5 in *A. castellanii* trophozoites and cysts.

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REFERENCES

1. Jung SY, Matin A, Kim KS, Khan NA. The capsule plays an important role in *Escherichia coli* K1 interactions with *Acanthamoeba*. Int J Parasitol 2007; 37: 417-423.
2. Khan NA. *Acanthamoeba*: biology and increasing importance in human health. FEMS Microbiol Rev 2006; 30: 564-595.
3. Marciano-Cabral F, Cabral G. *Acanthamoeba* spp. as agents of

disease in humans. Clin Microbiol Rev 2003; 16: 273-307.
4. Weekers PH, Bodelier PL, Wijen JP, Vogels GD. Effects of grazing by the free-living soil amoebae *Acanthamoeba castellanii*, *Acanthamoeba polyphaga*, and *Hartmannella vermiformis* on various bacteria. Appl Environ Microbiol 1993; 59: 2317-2319.
5. Allen PG, Dawidowicz EA. Phagocytosis in *Acanthamoeba*: I. A mannose receptor is responsible for the binding and phagocytosis of yeast. J Cell Physiol 1990; 145: 508-513.
6. Ghedin E, Fraser CM. A virus with big ambitions. Trends Microbiol 2005; 13: 56-57.
7. La Scola B, Audic S, Robert C, Jungang L, de Lamballerie X, Drancourt M, Birtles R, Claverie JM, Raoult D. A giant virus in amoebae. Science 2003; 299: 2033.
8. Alsam S, Jeong SR, Sissons J, Dudley R, Kim KS, Khan NA. *Escherichia coli* interactions with *Acanthamoeba*: a symbiosis with environmental and clinical implications. J Med Microbiol 2006; 55: 689-694.
9. Jung SY. Free living amoeba-bacteria interactions: analysis of *Escherichia coli* interactions with nonpathogenic or pathogenic free living amoeba. J Exp Biomed Sci 2011; 17: 7-12.
10. Rowbotham TJ. Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae. J Clin Pathol 1980; 33: 1179-1183.
11. La Scola B, Raoult D. Survival of *Coxiella burnetii* within free-living amoeba *Acanthamoeba castellanii*. Clin Microbiol Infect 2001; 7: 75-79.
12. Michel R, Burghardt H, Bergmann H. [*Acanthamoeba*, naturally intracellularly infected with *Pseudomonas aeruginosa*, after their isolation from a microbiologically contaminated drinking water system in a hospital]. Zentralbl Hyg Umweltmed 1995; 196: 532-544.
13. Thom S, Warhurst D, Drasar BS. Association of *Vibrio cholerae* with fresh water amoebae. J Med Microbiol 1992; 36: 303-306.
14. Winiecka-Krusnell J, Wreiber K, von Euler A, Engstrand L, Linder E. Free-living amoebae promote growth and survival of *Helicobacter pylori*. Scand J Infect Dis 2002; 34: 253-256.
15. Ly TM, Müller HE. Ingested *Listeria monocytogenes* survive and multiply in protozoa. J Med Microbiol 1990; 33: 51-54.
16. Barker J, Humphrey TJ, Brown MW. Survival of *Escherichia coli* O157 in a soil protozoan: implications for disease. FEMS Microbiol Lett 1999; 173: 291-295.
17. Steinert M, Birkness K, White E, Fields B, Quinn F. *Mycobacterium avium* bacilli grow saprozoically in coculture with *Acanthamoeba polyphaga* and survive within cyst walls. Appl Environ Microbiol 1998; 64: 2256-2261.
18. Siddiqui R, Malik H, Sagheer M, Jung SY, Khan NA. The type III secretion system is involved in *Escherichia coli* K1 interactions with *Acanthamoeba*. Exp Parasitol 2011; 128: 409-413.
19. Krishna-Prasad BN, Gupta SK. Preliminary report on engulfment and retention of *Mycobacterium* by trophozoites of axenically grown *Acanthamoeba castellanii* Douglas, 1930. Curr Sci 1978; 47: 245-247.
20. Cordingley JS, Wills RA, Villemez CL. Osmolarity is an independent trigger of *Acanthamoeba castellanii* differentiation. J Cell Biochem 1996; 61: 167-171.
21. Yan L, Cerny RL, Cirillo JD. Evidence that hsp90 is involved in the altered interactions of *Acanthamoeba castellanii* variants with bacteria. Eukaryot Cell 2004; 3: 567-578.
22. Gao LY, Harb OS, Abu Kwaik Y. Utilization of similar mechanisms by *Legionella pneumophila* to parasitize two evolutionarily distant host cells, mammalian macrophages and protozoa. Infect Immun 1997; 65: 4738-4746.
23. Sukumaran SK, Shimada H, Prasadarao NV. Entry and intracellular replication of *Escherichia coli* K1 in macrophages require expression of outer membrane protein A. Infect Immun 2003; 71: 5951-5961.
24. Harb OS, Venkataraman C, Haack BJ, Gao LY, Kwaik YA. Heterogeneity in the attachment and uptake mechanisms of the Legionnaires' disease bacterium, *Legionella pneumophila*, by protozoan hosts. Appl Environ Microbiol 1998; 64: 126-132.
25. Venkataraman C, Haack BJ, Bondada S, Abu Kwaik Y. Identification of a Gal/GalNAc lectin in the protozoan *Hartmannella vermiformis* as a potential receptor for attachment and invasion by the Legionnaires' disease bacterium. J Exp Med 1997; 186: 537-547.
26. Payne NR, Horwitz MA. Phagocytosis of *Legionella pneumophila* is mediated by human monocyte complement receptors. J Exp Med 1987; 166: 1377-1389.
27. Abu Kwaik Y. The phagosome containing *Legionella pneumophila* within the protozoan *Hartmannella vermiformis* is surrounded by the rough endoplasmic reticulum. Appl Environ Microbiol 1996; 62: 2022-2028.
28. Bozue JA, Johnson W. Interaction of *Legionella pneumophila* with *Acanthamoeba castellanii*: uptake by coiling phagocytosis and inhibition of phagosome-lysosome fusion. Infect Immun 1996; 64: 668-673.
29. Horwitz MA. The Legionnaires' disease bacterium (*Legionella pneumophila*) inhibits phagosome-lysosome fusion in human monocytes. J Exp Med 1983; 158: 2108-2126.
30. Swanson MS, Isberg RR. Formation of the *Legionella pneumophila* replicative phagosome. Infect Agents Dis 1993; 2: 224-226.