

# Interactions Between *Acanthamoeba culbertsoni* and Pathogenic Bacteria and their Inhibition by Lectin-Antibodies

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## Abstract

In this study, using pathogenic and non-pathogenic bacteria, it was analyzed whether a polyclonal serum and a monoclonal antibody to *A. culbertsoni* mannose-binding protein (MBP) could inhibit its interaction. The association of the amoeba with *E. coli* O157:H7 was very strong at a level of over 100%, but the non-pathogenic *E. coli* strain was about five times lower at 22%. Pathogenic *K. pneumoniae* also showed high association with amoeba by about 92% as compared with pathogenic *E. coli* O157:H7 and *S. agalactiae*. The polyclonal serum to MBP inhibited *E. coli* O157:H7 association to amoeba 2.5 times more than untreated *E. coli* O157:H7. Monoclonal antibody to MBP also inhibited bacterial association with amoeba but was not stronger than the polyclonal serum. Pathogenic *E. coli* O157:H7 showed about 88% invasion into amoeba and decreased about 22% as compared with associated *E. coli* O157:H7. Polyclonal serum to MBP inhibited about 55%, 50%, and 44% in *E. coli* O157:H7, *K. pneumoniae* and *S. agalactiae*, respectively. The invasion of *K. pneumoniae* and *S. agalactiae* was not high as polyclonal serum but was about 8% to 10% weaker than polyclonal serum. The pathogenic strains of *K. pneumoniae* and *S. agalactiae* showed less decrease in survival as shown at invasion than *E. coli* O157:H7 without antibody. This study provided the information that the pathogenic bacteria could be more interactive with *A. culbertsoni* trophozoites as a reservoir host than non-pathogenic *E. coli*, and the amoeba should interact with bacteria by the MBP lectin.

**Keywords:** *Acanthamoeba culbertsoni*, Association, Bacteria, Lectin, Invasion

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## INTRODUCTION

*Acanthamoeba* is a free-living protozoa isolated in a variety of environments, including air, soil, tap water and swimming pools. It is known to be one of the most ubiquitous protozoans<sup>1-3</sup>. The ability of *Acanthamoeba* to survive in such diverse environments is evidence that it can interact with bacteria, eat and live. *Acanthamoeba* acts as a reservoir for several pathogenic bacteria such as *Escherichia coli* K1 as an agent of meningitis<sup>4</sup>, *Legionella pneumophila* as an agent of Legionnaire's disease<sup>5</sup>, *Pseudomonas aeruginosa* as an agent of keratitis<sup>6</sup>, etc.

Environmental bacteria commonly colonize and form cluster such as biofilms, which generally contain a number of prokaryotic and eukaryotic cells associating with adhesion molecules and secreted compounds<sup>7</sup>. In addition, because the process of infection between amoebae and macrophages is very similar, amoeba is a powerful model for studying bacteria-macrophage interactions<sup>8,9</sup>. Their interactions were inferred from the phagocytic ability of *Acanthamoeba* and macrophage. Bacteria contain a polysaccharide layer, e.g., a capsule outside of the bacterial wall<sup>10</sup>. In a previous study, a polysaccharide glucuronoxylomannan composed of a capsule of *Cryptococcus neoformans* was ingested by *A. castellanii*, indicating an additional similarity between the mechanisms by which amoeba and phagocytes interact with cryptococcal components<sup>11</sup>. Moreover, receptors in *Acanthamoeba* are of interest in the interactions between amoeba and bacteria. One of those, mannose-binding protein (MBP) of *A. castellanii* and *A. culbertsoni*, was known to play an important role in the pathogenesis of infection by mediating the attachment of parasites to the host cells<sup>12,13</sup>. It may mediate the interaction of amoeba with other cells such as bacteria. In this study, using pathogenic and non-pathogenic bacteria such as *E. coli* O157:H7, *K. pneumoniae*, *S. agalactiae* and *E. coli* DH5 $\alpha$ , it was analyzed whether a polyclonal serum and a monoclonal antibody to MBP of *A. culbertsoni* could inhibit its interactions, such as bacterial association, invasion and survival. Moreover, the amount of difference between interactions were analyzed as compared with interaction untreated with antiserum or antibody.

## MATERIALS AND METHODS

### Amoeba culture

*A. culbertsoni* trophozoites (ATCC No. 30,171)<sup>14,15</sup> were cultured without shaking in 12 ml of PYG medium (0.75% proteose peptone (Kisan Bio, Seoul, Korea), 0.75% yeast extract (Kisan Bio, Seoul, Korea) and g1.5% glucose (Sigma-Aldrich Co., St. Louis, MO, USA)) in a T75 culture flask at 37°C. The media was refreshed 17-20 hours prior to experiments<sup>16,17</sup>. The result was more than 99% amoebae in the trophozoite form, which was subsequently used for carbohydrate selection.

### Bacterial strains

Three harmful bacteria, e.g., *E. coli* O157:H7 (ATCC No. 43895), *K. pneumoniae* (ATCC No. BAA-1705) and *S. agalactiae* (ATCC No. 13813), one non-pathogenic *E. coli* DH5 $\alpha$  (KCTC No. 22002) were applied for adhesion and invasion assay. *E. coli* strains and *K. pneumoniae* are rod-shaped but *S. agalactiae* exhibits a round form. The analysis of the two assays was also performed according to the different sized-bacteria. In this study, single colonies were subcultured into other tryptic soy agar (TSA, MB cell, Korea) plates at 37°C, and were checked by Gram-staining procedures<sup>18</sup>.

### MBP purification and production of its antibodies

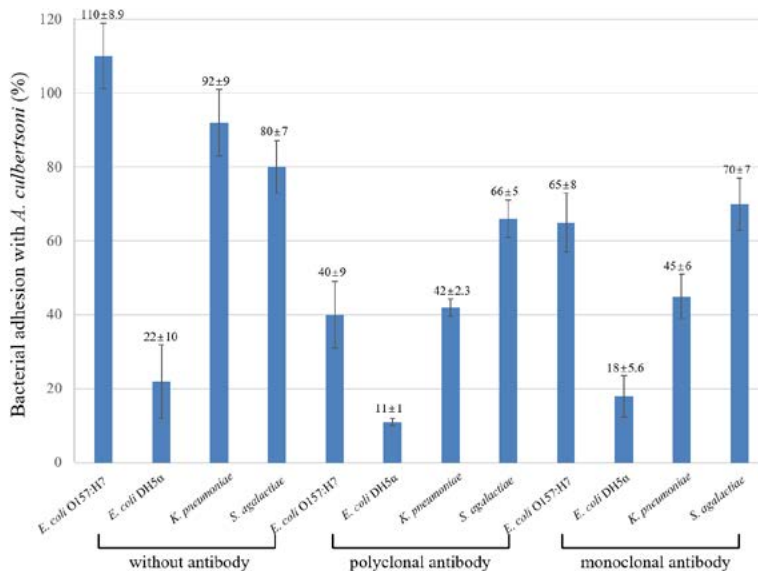
The protocols to purify MBP were described in the previous report<sup>13</sup>. Briefly, trophozoites of *A. culbertsoni* cultured in a T75 flask were washed three times with phosphate buffered saline (PBS) (Sigma-Aldrich Co., St. Louis, MO, USA), then centrifuged and dissolved with a lysis buffer containing with 50 mM Tris-HCl, 50 mM CaCl<sub>2</sub>, 150 mM NaCl, 1mM phenyl methanesulfonyl fluoride (PMSF), 2 mM  $\beta$ -mercapto ethanol, 0.5% CHAPS (Sigma-Aldrich Co., St. Louis, MO, USA) by a sonicator for 2 min with 20 W. The total amoebial lysate was collected by centrifugation (13,000 rpm, 1 h, 4°C) and purified with  $\alpha$ -D-mannose agarose (Sigma-Aldrich Co., St. Louis, Missouri, USA) affinity column (Qiagen, CA, USA). Unbound lysate was removed with the washing buffer and the bound lysate were eluted with 150 mM mannose<sup>12,19,20</sup>. The eluates were biweekly immunized into BALB/C mice (Narabiotech, Pyeongtaek, South Korea) three times for six weeks, and then polyclonal serum was isolated. The monoclonal antibody, the selected hybridomas clones kindly provided

from the previous study<sup>13</sup>, were moved into a T75 culture flask. The monoclonal antibody was a IgM class of kappa chain named DG11.

Assay of amoeba-bacteria interactions *Acanthamoeba* can ingest bacteria such as *E. coli*, *S. aureus*, *Pseudomonas aeruginosa*, etc. as a reservoir host. Prior to ingestion in the cytoplasm of *Acanthamoeba*, bacteria can adhere to the outer membrane of the amoeba and then enter its cytoplasm. The processes of bacterial adherence and entrance were called association and invasion, respectively. Their experimental procedures were referred by Jung et al., 2007<sup>3</sup> and modified a little. Briefly, *A. culbertsoni* trophozoites were cultured in 24-well culture plates with PYG medium. *Acanthamoeba* was incubated with each bacterium ( $2 \times 10^5$  cfu/0.5 ml of PBS) and plates incubated for 1 h at room temperature (RT). Obtained single colonies were diluted with 0.85% NaCl and were set to 0.5 of McFaland turbidity producing  $1.5 \times 10^3$  to  $1.5 \times 10^6$  colony forming units (CFU)/ml<sup>21</sup>. After the incubation, amoebae were washed 3 times with PBS to remove unassociated bacteria, and amoebae were counted using a haemocytometer. Finally, amoebae were dissolved by adding SDS

(0.5% final concentration) to each well for 30 mi and the number of bacteria was enumerated by plating on nutrient agar plates<sup>4</sup>. The percentage of bacterial association was calculated as follows: recovered bacteria (cfu)/total bacteria (cfu)  $\times$  100 = % bacteria associated with *Acanthamoeba*.

Invasion assay represented entrance of bacteria into the cytoplasm of *A. culbertsoni* trophozoites. For the invasion assay, after the bacteria were incubated with amoebae, gentamicin antibiotics was added for 45 min (100  $\mu$ g/ml, final concentration) to remove extracellular bacteria. Finally, amoebae were counted and the intracellular bacteria were also counted. The percentage of bacterial invasion was calculated as follows: recovered bacteria (cfu)/total bacteria (cfu)  $\times$  100 = percentage of intracellular bacteria. For these association and invasion assays, the polyclonal serum or monoclonal antibody of DG11 to MBP was preincubated with *A. culbertsoni* trophozoites for 30 min prior to the assays and then incubated with bacteria. Invaded bacteria can survive within the amoeba cytoplasm and vacuoles. The invaded bacteria treated with gentamicin were allowed to survive within the



**Fig. 1.** Percentage of bacterial association with *A. culbertsoni* trophozoites. Pathogenic *E. coli* O157:H7, *K. pneumoniae*, *S. agalactiae* and one non-pathogenic *E. coli* DH5α were analyzed by association, and antibodies to MBP was preincubated with the amoeba for 30 min prior to the association assay. This experiment was performed in triplicate wells with three times, and data were described by mean  $\pm$  standard deviation (SD) value.

amoeba cytoplasm for 1 hour, and the surviving bacteria were calculated as mentioned above.

**Statistical analysis**

Statistical differences between groups or samples were determined by using a Student two-sample *t* test. The difference was considered significant when *P* was <0.05.

**RESULTS**

**Analysis of bacterial association with amoeba trophozoites**

To understand how many bacteria could adhere to *A. culbertsoni* trophozoites, an association assay was performed. *A. culbertsoni* trophozoites can possess or form a lot of pseudopodia which can associate with bacteria. Also, bacteria have various factors that associate with *A. culbertsoni*, e.g., OmpA, LPS, etc. MBP<sup>3</sup>. MBP in *A. culbertsoni* trophozoites were one of the association factors<sup>13</sup>, implying that antibodies would inhibit their association. The association of *E. coli* O157:H7 with amoeba was extremely strong at over 100%, but non-pathogenic *E. coli* strain was about five times lower of 22% (Fig. 1). Another pathogenic strain, *K. pneumoniae*, also showed high association with amoeba by

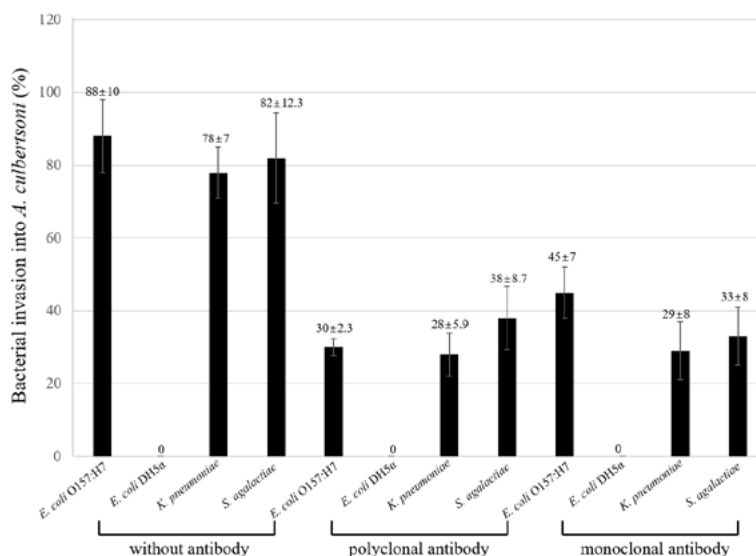
about 92% as compared with pathogenic *E. coli* O157:H7. *S. agalactiae* showing a round form of chains also had a high association with amoeba by about 80%. Taken together, it suggested that pathogenic strains of bacteria could associate with *A. culbertsoni* trophozoites four or five times higher than non-pathogenic *E. coli*.

**Effect of MBP antibodies for amoeba interactions with bacteria**

The polyclonal serum to MBP inhibited *E. coli* O157:H7 association to amoeba 2.5 times more than untreated *E. coli* O157:H7. The other three bacteria also inhibited their association with amoeba irrespective of bacterial pathogenicity. On the other hand, monoclonal antibody to MBP also inhibited bacterial association with amoeba as compared with antibody-untreated amoeba but not stronger than the polyclonal serum. Because of the polyclonal serum more antibodies recognized various epitopes, and the monoclonal antibody to only MBP would not show strong inhibition as compared with the polyclonal serum.

**Bacterial invasion by the MBP antibodies**

Amoebae have the potential to ingest the associated or adhered bacteria because bacteria are small-sized, and amoebae express pseudopodia



**Fig. 2.** Percentage of bacterial invasion into *A. culbertsoni* trophozoites. Pathogenic *E. coli* O157:H7, *K. pneumoniae*, *S. agalactiae* and one non-pathogenic *E. coli* DH5α were analyzed by invasion. Invasion assay was done post to the association assay above. This experiment was performed in triplicate wells with three times, and data were described by mean ± standard deviation (SD) value.

to eat bacteria<sup>1</sup>. Here, it was analyzed how much of a percentage of bacteria could invade the amoeba and also the polyclonal serum and monoclonal antibody above inhibited bacterial invasion by the treatment with the antibiotic gentamicin which could kill uninvaded remaining bacteria (Fig. 2). Bacterial invasion showed very similar patterns as the association results in Fig. 1, but the number of bacteria invading decreased. Pathogenic *E. coli* O157:H7 showed about 88% of invasion into amoeba and decreased about 22% as compared with associated *E. coli* O157:H7. On the other hand, associated non-pathogenic *E. coli* DH5 $\alpha$  with amoeba was about 22% but never invaded into amoebae. Polyclonal serum to MBP inhibited about 55%, 50%, and 44% in *E. coli* O157:H7, *K. pneumoniae* and *S. agalactiae*, respectively. The inhibitory effect of the monoclonal antibody was not as high as the polyclonal serum, but *E. coli* O157:H7 invasion was inhibited about 43% as compared with untreated *E. coli* O157:H7, but not inhibited 15% as compared with polyclonal serum. The invasion of *K. pneumoniae* and *S. agalactiae* was not as high as polyclonal serum but about 8% to 10% was weaker than polyclonal serum.

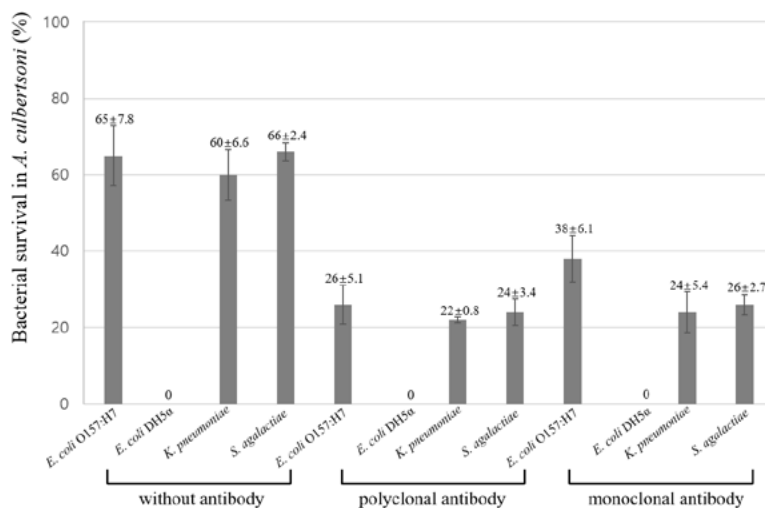
**Bacterial survival in the amoeba and the effect of MBP antibodies**

The invaded bacteria were subsequently

incubated for one hour for survival within amoeba cytoplasm. The surviving bacteria were calculated as mentioned at association and invasion assay (Fig. 3). Overall, survived bacteria were decreased as compared with invasion results, suggesting that some bacteria were killed within amoeba cytoplasm. Interestingly, the pathogenic strains of *K. pneumoniae* and *S. agalactiae* showed less decrease in survival as shown at invasion than *E. coli* O157:H7 without antibody, suggesting that pathogenicity should be an important factor in survival. Moreover, the polyclonal and monoclonal antibody showed similar decreasing patterns.

**DISCUSSION**

Bacteria can interact and communicate with protozoan parasites, e.g., *Acanthamoeba* by receptor-ligand binding. Outer-membrane protein A (OmpA) and lipopolysaccharide (LPS) were important bacterial factors responsible for the interaction between *A. castellanii* and pathogenic *E. coli* K1<sup>4</sup>. On the other hand, an interesting molecule associated with the contact-dependent pathway was MBP in *A. castellanii* which had an effect on the contact-dependent cytotoxicity to host cells<sup>12,22</sup>. It was clear that MBP in *A. castellanii* interacted with alpha-mannose of saccharides in bacteria. The monoclonal antibody to MBP in *A.*



**Fig. 3.** Percentage of bacterial survival in *A. culbertsoni* trophozoites. Bacteria incubated with gentamicin were allowed to survive within the amoebae. Survival assay was done post to the invasion assay above. This experiment was performed in triplicate wells with three times, and data were described by mean  $\pm$  standard deviation (SD) value.

*culbertsoni*, DG11 of IgM of kappa chains detected 83 kDa on gels was produced<sup>23</sup>. It was applied to analyze the interaction between *A. culbertsoni* trophozoites and several bacteria in this study. The MBP had very important aspects because the MBP was expressed higher in the movement which could be done by pseudopodia, implying that the MBP could be one of the crucial factors in their interactions.

Three pathogenic bacteria such as *E. coli* O157:H7, *K. pneumoniae*, *S. agalactiae* and one non-pathogenic *E. coli* DH5 $\alpha$  showed different patterns of bacterial interactions with *A. culbertsoni* trophozoites and their interactions were inhibited by the pre-incubation of polyclonal serum and monoclonal antibody to MBP into *A. culbertsoni*. According to Alsam et al., 2006<sup>4</sup>, the interaction between *E. coli* K1 and *A. castellanii* was regulated by the pathogenicity of *E. coli*. Similar patterns were observed in this study as pathogenic *E. coli* O157:H7, *K. pneumoniae*, *S. agalactiae* were higher in the interactions than a non-pathogenic strain. The size of bacteria varies around 0.5 to 2  $\mu$ m and the amoeba has the size of up to 25  $\mu$ m. Pathogenic *E. coli* O157:H7 and *K. pneumoniae* are generally bigger than *S. agalactiae* even though *S. agalactiae* forms a long chain. Our results showed that there was not significant difference between rod type and long coccus type by size. Amoeba showed the result of bacteria predation through interaction with bacteria. However, like macrophages, the exact mechanism of bacterial predation within amoeba, such as autophagy, is not yet known, but protein-protein interactions will analyze similar autophagy functions in amoeba<sup>23</sup>. The results showed that pathogenic *E. coli* O157:H7 survived in amoeba by about 65%. Since amoeba can eat and live with bacteria, bacteria can exist for a long time in amoeba. Bacteria and fungi are still present in women with abortions<sup>24</sup>. In particular, about 14% of *Ureaplasma parvum* was detected by PCR. Since macrophages have the ability to phagocytose bacteria, the interaction of bacteria, such as amoeba and *U. parvum*, used in this experiment instead of macrophages, may be helpful in studying bacterial survival. This study provided the information that the pathogenic bacteria could be more interactive with *A. culbertsoni* trophozoites as a reservoir host than non-pathogenic *E. coli*

DH5 $\alpha$ , and the amoeba should interact with bacteria by the MBP lectin.

In this project by using pathogenic and non-pathogenic bacteria such as *E. coli* O157:H7, *K. pneumoniae*, *S. agalactiae* and *E. coli* DH5 $\alpha$ , it was analyzed whether a polyclonal serum and a monoclonal antibody to MBP of *A. culbertsoni* could inhibit its interaction such as bacterial association, invasion and survival. Moreover, it was analyzed that how much interaction was different when compared with interaction untreated with antiserum or antibody. We demonstrated that pathogenic bacteria could be more interactive with *A. culbertsoni* trophozoites as a reservoir host than non-pathogenic *E. coli* DH5 $\alpha$ , and the amoeba should interact with bacteria by the MBP lectin.

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#### ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

#### DATA AVAILABILITY

All datasets generated or analyzed during the study are included in the manuscript.

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