Inhibition of interactions between *Acanthamoeba culbertsoni* trophozoites and bacteria by antibodies to a mannose-binding protein

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ABSTRACT

Introduction and Aim: *Acanthamoeba* acts as a reservoir for a variety of bacterial pathogens including *Escherichia coli K1*, *Legionella pneumophila*, etc., In this study, it was analyzed whether a polyclonal serum and a monoclonal antibody to *Acanthamoeba culbertsoni* mannose-binding protein (MBP) could inhibit its interactions such as bacterial association, invasion and survival.

Materials and Methods: In our findings, the amoeba was highly associated with *E. coli* O157:H7 by about 97%, but a non-pathogenic strain of *E. coli* DH5α was associated three-times lower than the pathogenic *E. coli* O157:H7. On the other hand, the association of *Staphylococcus aureus* and *Bacillus subtilis* with the amoeba was about 60% and 65%, respectively. The polyclonal serum to MBP inhibited amoebial association with the four above said bacteria by about 35% to 40% except for non-pathogenic *E. coli* DH5α which had a 28% decrease as compared with untreated *E. coli* DH5α. On the other hand, monoclonal antibody to MBP also decreased the amoebial inhibition with bacteria, but its inhibitory effect was not as high as the polyclonal serum. After bacteria associate with the amoeba, they can be ingested or invaded into the amoeba. *S. aureus* and *B. subtilis* were not higher than *E. coli* O157:H7 but 18% of the bacteria invaded the amoeba. When the polyclonal serum was preincubated with the amoeba, it inhibited the bacterial invasion of about 19% in *E. coli* O157:H7. On the other hand, *S. aureus* and *B. subtilis* were inhibited by 12% and 10% by the polyclonal serum, respectively. The inhibitory effect of the monoclonal antibody was not as high as the polyclonal serum, but *E. coli* O157:H7 invasion was inhibited by about 13% as compared with untreated *E. coli* O157:H7. The invaded bacteria were subsequently incubated for one hour for survival within amoeba cytoplasm.

Results: Surviving bacteria were decreased as compared with invasion results and the decrease percentage of survived *E. coli* O157:H7 was about 21% as compared with invaded bacteria. However, the other two bacteria, *S. aureus* and *B. subtilis*, showed less decrease than the invaded bacteria. On the other hand, the polyclonal and monoclonal antibody showed similar decreasing patterns.

Conclusion: Therefore, MBP was one of the mediators for crosstalk between *A. culbertsoni* trophozoites and bacteria. This study will be helpful for understanding interactions between the amoeba and bacteria via lectins, e.g., MBP. Furthermore, with characteristics of *A. culbertsoni* to phagocytose bacteria, MBP could be one of the very important factors for crosstalk and the understanding of pathophysiology.

Keywords: Mannose-binding protein; bacteria; *Acanthamoeba*.

INTRODUCTION

Acanthamoeba culbertsoni is a causative agent of granulomatous amoebic encephalitis (GAE), a condition that predominantly occurs in immuno compromised individuals which are typically fatal (1, 2). *A. culbertsoni* causes GAE (3) by triggering cell necrosis, as it causes cytopathic effects on target cells, such as contractions, vesiculation, and nuclear condensation (4). *Acanthamoeba* trophozoites destroy nerve cells by contact-dependent cytolysis and by the ingestion of nerve cells through amoebastomes (5).

Acanthamoeba acts as a reservoir for a variety of bacterial pathogens including *E. coli* K1 (a causative agent of meningitis) (6-8), *Legionella pneumophila* (a causative agent of Legionnaire's disease) (9) and may act as vectors to transmit these pathogens to susceptible hosts. Most bacteria contain some sort of polysaccharide layer, e.g., a capsule outside of the cell wall or outer membrane. Invasive *E. coli* K1 interacted with *A. castellanii* trophozoites more than non-invasive *E. coli* K12, which indicate more association, invasion and survival of *E. coli* K1 with *A. castellanii* (6, 8).

It has been reported that a mannose-binding protein in *A. culbertsoni* would be a very important factor in the interaction of an amoeba and a target cell (10). Moreover, the monoclonal antibody of DG11 to MBP inhibited the phagocytosis of *A. culbertsoni* trophozoites.

In this study, it was analyzed whether a polyclonal serum and a monoclonal antibody to *A. culbertsoni* MBP could inhibit its interaction such as bacterial association, invasion, and survival. Moreover, it was

also analyzed how different interaction were when compared with interactions untreated with antiserum or antibody.

MATERIALS AND METHODS

Culture of *A. culbertsoni* trophozoites and purification of its MBP

A. culbertsoni trophozoites (ATCC No. 30, 171; (11-12) were grown without shaking in 12 ml of PYG medium (proteose peptone 0.75% (w/v) (Kisan Bio, Seoul, Korea), yeast extract 0.75% (w/v) (Kisan Bio, Seoul, Korea) and glucose 1.5% (w/v) (Sigma-Aldrich Co., St. Louis, MO, USA)) in a 75T culture flask at 37°C, and the media was refreshed 17-20 hours prior to experiments as previously described (13-14). This resulted in more than 99% amoebae in the trophozoite form, which were subsequently used for carbohydrate selections.

For the purification of a mannose-binding protein (MBP) of *A. culbertsoni* trophozoites, *A. culbertsoni* trophozoites were washed with phosphate-buffered saline (PBS) (Sigma-Aldrich Co., St. Louis, MO, USA) three times, and after centrifugation, were then lysed with a lysis buffer (50 mM Tris-HCl, 50 mM $CaCl_2$, 150 mM $NaCl_2$, 1 mM phenylmethane sulfonyl fluoride (PMSF), 2 mM β-mercaptoethanol, 0.5% CHAPS) (Sigma-Aldrich Co., St. Louis, MO, USA) using a sonicator (20 W, total 2 min) (15). The amoeba lysates were purified by centrifugation (13,000 rpm, 1 h, 4°C) and were chromatographed on an α-D-mannose agarose (Sigma-Aldrich Co., St. Louis, MO, USA) affinity column (Qiagen, CA, USA). The unbound components were removed by washing buffer with the elution buffer and bound components were eluted by 1 ml of 150 mM mannose (16-18).

The polyclonal serum and monoclonal antibody to the MBP were obtained from our previous research (10). Briefly, for the monoclonal antibody, the selected hybridoma clones were transferred into a 75T flask culture flask. It represented an IgM class of kappa chain analyzed from the supernatant and was named with DG11.

Culture of bacteria

Two Gram-positive bacteria and two Gram-negative bacteria were applied. Briefly, *E. coli* O157: H7 (ATCC No. 43895), non-pathogenic *E. coli* DH5α (KCTC No. 22002), *Staphylococcus aureus* (ATCC No. 25923) and *Bacillus subtilis* (KCTC No. 1104) strains were applied to test association with and invasion into *A. culbertsoni* trophozoites. In this study, the bacteria were not divided by characteristics of diseases but simply described as human infections. Single colonies were sub-cultured into other tryptic soy agar (TSA, MB cell, Korea) plates at 37℃, and were double-checked by Gram-staining procedures (19).

Association, invasion assay, and survival assay

Acanthamoeba can ingest bacteria such as *E. coli*, *S. aureus*, *P. aeruginosa*, etc., 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 are called association, invasion, and survival. Their experimental procedures were referred to by Jung *et al.*, (8) and modified a little. Briefly, *A. culbertsoni* trophozoites were cultured in 24-well plates in PYG medium. *Acanthamoeba* was incubated with each bacterium (2 × 106 cfu /well/0.5 ml of PBS) and plates were incubated for up to 1 h at room temperature. To culture accurate colonies, the obtained single colonies were diluted with 0.85% NaCl and were adjusted into 0.5 of McFaland turbidity, which could produce about 1.5 × 103 to 1.5 × 106 colony forming units (CFU)/ml (20). Following this incubation, amoebae were washed with PBS three times to remove non-associated bacteria and amoebae were counted using a haemocytometer. Finally, amoebae were lysed by adding SDS (0.5% final concentration) to each well for 30 min and the numbers of bacteria were enumerated by plating on nutrient agar plates as previously described (6). The percentage of bacterial association was calculated as follows: recovered bacteria (cfu)/total bacteria (cfu) × 100 = % bacteria associated with *Acanthamoeba*. For the invasion assay, after the bacteria were incubated with amoebae, the gentamicin of antibiotics was added for 45 min (100 μg/ml, final concentration) to kill extracellular bacteria. Finally, amoebae were counted, and intracellular bacteria were enumerated as described above. The percentage of bacterial invasion was calculated as follows: recovered bacteria (cfu)/total bacteria (cfu) × 100 = % intracellular bacteria. Invaded bacteria can survive within the amoeba's cytoplasm and vacuoles. In our experiment, the invaded bacteria treated with gentamicin were allowed to survive within the amoeba cytoplasm for 1 hour, and the surviving bacteria were calculated as mentioned above. For these associations, invasion assays, and survival assays, the monoclonal antibody of DG11 to MBP was preincubated with *A. culbertsoni* trophozoites for 30 minutes prior to the assays and then incubated with bacteria.

RESULTS

Association of *A. culbertsoni* trophozoites with bacteria and the effect of the monoclonal antibody to MBP

To understand whether a monoclonal antibody to MBP played a role to associate *A. culbertsoni* trophozoites with bacteria, an association assay was performed. The association is a crucial step to interact the amoeba with the bacteria, and then the amoeba can ingest the bacteria. The inhibition of amoebial phagocytosis was observed by the addition

of the antibody in the previous study (10). In our findings, the amoeba was highly associated with *E. coli* O157: H7 by about 97%, but the non-pathogenic strain of *E. coli* DH5α was associated three-times lower than the pathogenic *E. coli* O157: H7 (Fig. 1). On the other hand, the association of *S. aureus* and *B. subtilis* with the amoeba was about 60% and 65%, respectively.

The polyclonal serum to MBP inhibited amoebial association with the four bacteria above by about 35% to 40% except for non-pathogenic *E. coli* DH5α of 28% decrease as compared with untreated *E. coli* DH5α. On the other hand, a monoclonal antibody to MBP also decreased the amoebial inhibition with bacteria, but its inhibitory effect was not as high as the polyclonal serum. The monoclonal antibody to MBP inhibited amoebial association with the four bacteria above by about 12% to 27% as compared with untreated bacteria. The monoclonal antibody was inhibited less than the polyclonal serum by about 13% to 18%.

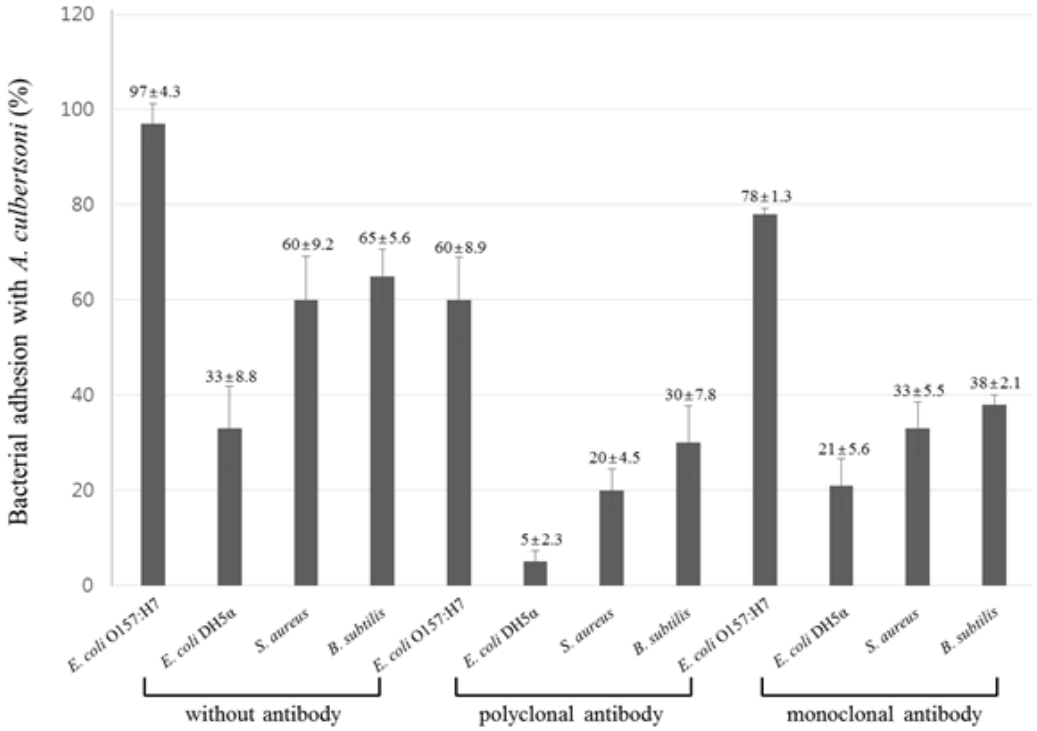

Fig. 1: Percentage of bacterial association with *A. culbertsoni* trophozoites. Four kinds of bacteria were applied to understand their association, and antibodies to MBP was preincubated with the amoeba for 30 min prior to the association assay. This experiment was performed in triplicate wells with three times, and data were described by mean ± standard deviation (SD) value.

Invasion into and survival of bacteria in *A. culbertsoni* trophozoites and the effect of the monoclonal antibody to MBP

After bacteria associate with the amoeba, they can be ingested or invade the amoeba. Here, the percentage of bacteria that could invade into the amoeba and also the polyclonal serum and monoclonal antibody above was analyzed and inhibited bacterial invasion by the treatment of gentamicin of antibiotics (Fig. 2). Non-pathogenic *E. coli* DH5α did not invade the amoeba, but *E. coli* O157: H7 invaded the amoeba by 61%. *S. aureus* and *B. subtilis* were not higher than *E. coli* O157: H7 but 18% of the bacteria invaded the amoeba. When the polyclonal serum was preincubated with the amoeba, it inhibited the bacterial invasion of about 19% in *E. coli* O157: H7. On the other hand, *S. aureus* and *B. subtilis* were inhibited by 12% and 10% by the polyclonal serum, respectively. The inhibitory effect of the monoclonal antibody was not as high as the polyclonal serum, but *E. coli* O157: H7 invasion was inhibited by about 13% as compared with untreated *E. coli* O157: H7. The invasion of *S. aureus* and *B. subtilis* were inhibited by about 10% and 2% by the monoclonal antibody as compared with untreated *E. coli* O157: H7. The invaded bacteria were subsequently incubated for one hour for survival within amoeba cytoplasm. The survived bacteria were calculated as mentioned at association and invasion assay (Fig. 3). Overall, surviving bacteria were decreased as compared with invasion results, suggesting that some bacteria were killed within the amoeba cytoplasm. Interestingly, the decrease percentage of survived *E. coli* O157: H7 was about 21% as compared with invaded bacteria, but the other two bacteria, *S. aureus* and *B. subtilis*, showed less decrease than the invaded bacteria. On the other hand, the polyclonal and monoclonal antibody showed similar decreasing patterns.

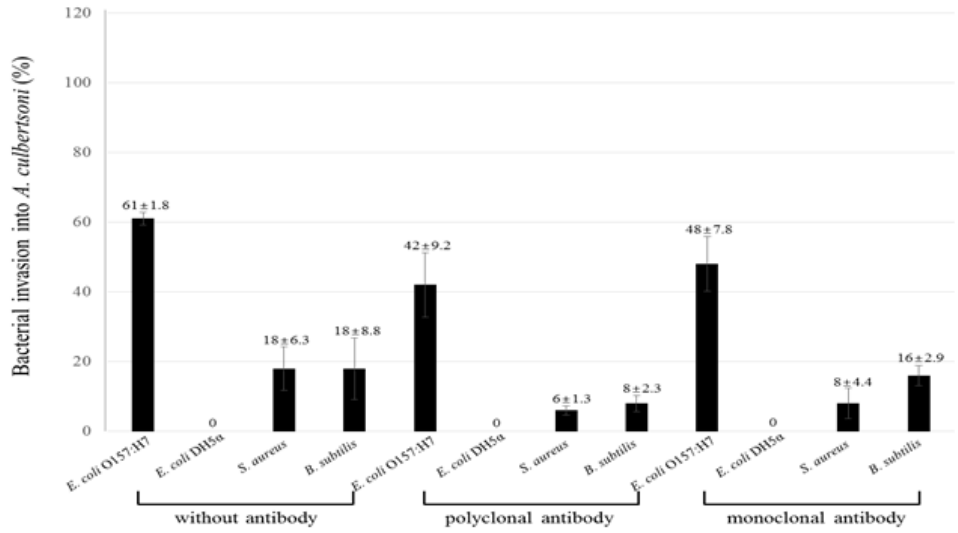

Fig. 2: Percentage of bacterial invasion into *A. culbertsoni* trophozoites. Four kinds of bacteria were applied to understand their invasion. Invasion assay was done post to the association assay above. This experiment was performed in triplicate wells with three times, and data were described by mean ± standard deviation (SD) value.

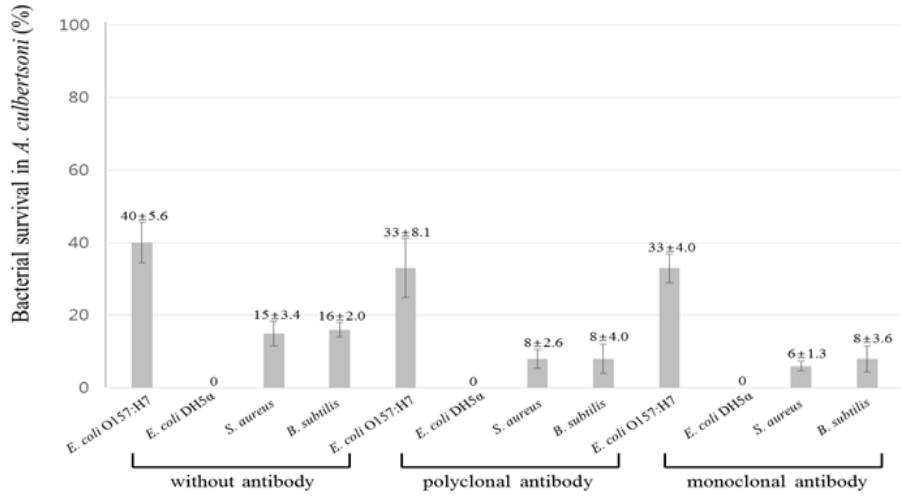

Fig. 3: Percentage of bacterial survival in *A. culbertsoni* trophozoites. Bacteria incubated with gentamicin were allowed to survive within the amoebae. Survival assay was done post to the invasion assay above. This experiment was performed in triplicate wells with three times, and data were described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

DISCUSSION

One of the molecules associated with the contact-dependent pathway was the mannose-binding protein (MBP) in *A. castellanii* which played an important role in the contact-dependent cytotoxicity to host cells (15-16). In the previous study, the monoclonal antibody to MBP, DG11 of IgM of kappa chains detected 83 kDa on gels (10). When the amoeba moved forward, the MBP was concentrated in the direction of its movement. Taken together, it implied that MBP would be one of the factors associated with the interaction, motility, and phagocytosis.

In this study, *A. culbertsoni* trophozoites act as a reservoir for bacterial pathogens clearly associated with bacteria, and the bacteria invaded and survived within the amoeba. *Acanthamoeba* resembles human macrophages in many ways, particularly in their phagocytic activity and cell surface receptors (21) and in those macrophages and *Acanthamoeba* exhibit parallel mechanisms in their interactions with *L. pneumophila* (22), suggesting that MBP would be also strong factor to interact with or phagocytose bacteria.

In this study, amoebial association with a pathogenic strain of *E. coli* O157: H7 and bacterial invasion into the amoeba was higher than the non-pathogenic strain of *E. coli* DH5α. There was no huge difference, but the association and invasion of pathogenic *E. coli* O157: H7 was higher than an opportunistic strain of *S. aureus*. Interestingly, *B. subtilis* is known as a

generally non-pathogenic strain, but not the same as the non-pathogenic strain of *E. coli* DH5α, similarly associated with the amoeba and invaded into the amoeba. In the previous study, pathogenic *E. coli* K1 interacted with amoebae at a higher percentage than non-pathogenic laboratorial *E. coli* K12 (8). Subsequent bacteria incubated for one hour survived within the amoeba even though some bacteria were killed. Surviving bacteria decreased as compared with invasion results and the polyclonal and monoclonal antibody showed similar decreasing patterns. In particular, the polyclonal serum and monoclonal antibody to MBP showed the inhibition of association and invasion but had no significant difference. MBP is related with motility (10).

CONCLUSION

A. culbertsoni trophozoites can ingest bacteria by MBP of motility-associated factors, e.g., cytoskeletons, suggesting that MBP play an important role in the interaction with bacteria rather than other proteins. Therefore, MBP was one of the mediators to cross talk between *A. culbertsoni* trophozoites and bacteria. This study will be helpful to understand interactions between the amoeba and bacteria via lectins, e.g., MBP. Furthermore, with characteristics of *A. culbertsoni* to phagocytose bacteria, MBP would be one of the very important factors for cross talk and the understanding of pathophysiology.

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CONFLICT OF INTEREST: The author has no conflict of interest.

REFERENCES

1. Schuster, F. L., Visvesvara, G. S. Free-living amoebae as opportunistic and non-opportunistic pathogens of humans and animals. Int J Parasitol. 2004; 34(9): 1001-1027.
2. Walochnik, J., Aichelburg, A., Assadian, O., Steuer, A., Visvesvar, a G., Vetter, N., *et al*. Granulomatous amoebic encephalitis caused by *Acanthamoeba* amoebae of genotype T2 in a human immunodeficiency virus-negative patient. J Clin Microbiol. 2008; 46(1): 338-378.
3. Martinez, A. J. Infection of the central nervous system due to *Acanthamoeba*. Rev Infect Dis. 1991; 13: 399-402.
4. Shin, H. J., Cho, M. S., Jung, S. Y., Kim, H. I., Park, S., Seo, J. H., *et al*. Cytopathic changes in rat microglial cells induced by pathogenic *Acanthamoeba culbertsoni*: morphology and cytokine release. Clin Diag Lab Immunol. 2001; 8(4): 837-840.
5. Pettit, D. A. D, Williamson, J., Cabral, G. A., Marciano-Cabral, F. In vitro destruction of nerve cell cultures by *Acanthamoeba* spp. A transmission and scanning electron microscopy study. J Parasitol. 1996; 82(5): 769-777.
6. Alsam, S., Jeong, S. R., Sissons, J., Dudley, R., Kim, K. S., Khan, N. A. *Escherichia coli* interactions with *Acanthamoeba*: a symbiosis with environmental and clinical implications. J Med Microbiol. 2006; 55(6): 689-694.
7. Jung, S. Y. Free living amoeba-bacteria interactions: Analysis of *Escherichia coli* interactions with nonpathogenic or pathogenic free-living amoeba. J Exp Biomed Sci. 2011; 17(1): 7-12.
8. Jung, S. Y., Matin, A., Kim, K. S., Khan, N. A. The capsule plays an important role in *Escherichia coli* K1 interactions with *Acanthamoeba*. Int J Parasitol. 2007; 37(3-4): 417-423.
9. Rowbotham, T. J. Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae. J Clin Pathol 1980; 33(12): 1179-1183.
10. Kang, A. Y., Park, A. Y., Shin, H. J., Khan, N. A., Maciver, S. K., Jung, S. Y. Production of a monoclonal antibody against a mannose-binding protein of *Acanthamoeba culbertsoni* and its localization. Exp Parasitol. 2018; 192; 19-24.
11. Kim, Y. K., Kim, T. U., Joung, I. S., Im, K. I. A comparative study on hydrolase activities in *Acanthamoeba culbertsoni* and *A. royreba*. Korean J Parasitol. 1988; 26(2): 95-106.
12. Kong, H. H., Seo, S. A., Shin, C. O., Im, K. I. The effect of immunization with *Acanthamoeba culbertsoni* in mice born to immune mother. Korean J Parasitol. 1993; 31(2): 157-163.
13. Krishna-Prasad, B. N., Gupta, S. K. Preliminary report on engulfment and retention of Mycobacteria by trophozoites of axenically grown *Acanthamoeba castellanii* Douglas. Curr Sci. 1978; 47(7): 245-247.
14. Yoo, K. T., Jung, S. Y. Effects of mannose on pathogenesis of *Acanthamoeba castellanii*. Korean J Parasitol. 2012; 50(4): 365-369.
15. Garate, M., Cao, Z., Bateman, E., Panjwani, N. Cloning and characterization of a novel mannose-binding protein of *Acanthamoeba*. J Biol Chem. 2004; 279(28): 29849-29905.
16. Kim, J. H., Lee, J., Sohn, H. J., Song, H. O., Kim, J. Y., Lee, W. J., *et al.* Production of monoclonal antibodies for *Plasmodium vivax* lactate dehydrogenase and patient sera screening using sandwich ELISA. Parasitol Res. 2012; 111(4): 1645-1650.
17. Kim, J. H., Matin, A., Shin, H. J., Park, H., Yoo, K. T., Yuan, X. Z., *et al*. Functional roles of mannose-binding protein in the adhesion, cytotoxicity and phagocytosis of *Acanthamoeba castellanii*. Exp Parasitol. 2012; 132(2): 287-292.
18. Garate, M., Cubillos, I., Marchant, J., Panjwani, N. Biochemical characterization and functional studies of *Acanthamoeba* mannose-binding protein. Infect Immun. 2005; 73(9): 5775-5781.
19. Lim, K. B., Boey, L. P., Khatijah, M. Gram's-stained microscopy in the etiological diagnosis of *Malassezia (Pityrosporon) folliculitis*. Arch Dermatol. 1988; 124(4): 492.
20. Song, K. J., Jung, S. Y. Biocidal effects of chlorine dioxide on isolated and identified pathogens from nosocomial environment–biochemical and technical convergence. J Digit Converg. 2017; 15(6): 339-344.
21. Yan, L., Cerny, R. L., Cirillo, J. D. Evidence that hsp90 is involved in the altered interactions of *Acanthamoeba castellanii* variants with bacteria. Eukaryot Cells. 2004; 3(3): 567-578.
22. Gao, L. Y., Harb, O. S., 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(11): 4738-4746.