Specific Detection of *Acanthamoeba* species using Polyclonal Peptide Antibody Targeting the Periplasmic Binding Protein of *A. castellanii*

Min-Jeong Kim, Fu-Shi Quan, Hyun-Hee Kong, Jong-Hyun Kim, Eun-Kyung Moon

Abstract: *Acanthamoeba* keratitis (AK) is a rare ocular disease, but it is a painful and sight-threatening infectious disease. Early diagnosis and adequate treatment are necessary to prevent serious complications. While AK is frequently diagnosed via several PCR assays or *Acanthamoeba*-specific antibodies, a more specific and effective diagnostic method is required. This study described the production of a polyclonal peptide antibody against the periplasmic binding protein (PBP) of *A. castellanii* and investigated its diagnostic potential. Western blot analysis showed that the PBP antibody specifically reacted with the cell lysates of *A. castellanii*. However, the PBP antibody did not interact with human corneal epithelial (HCE) cells and the other 3 major causative agents of keratitis. Immunocytochemistry (ICC) results revealed the specific detection of *A. castellanii* trophozoites and cysts by PBP antibodies when *A. castellanii* were co-cultured with HCE cells. PBP antibody specificity was further confirmed by co-culture of *A. castellanii* trophozoites with *F. solani*, *S. aureus*, and *P. aeruginosa* via ICC. The PBP antibody specifically reacted with the trophozoites and cysts of *A. polyphaga*, *A. hatchetti*, *A. culbertsoni*, *A. royleba*, and *A. healyi*, thus demonstrated its genus-specific nature. These results showed that the PBP polyclonal peptide antibody of *A. castellanii* could specifically detect several species of *Acanthamoeba*, contributing to the development of an effective antibody-based AK diagnostics.

Key words: *Acanthamoeba* keratitis, periplasmic binding protein, peptide antibody, species specificity
distinguish *Acanthamoeba* spp. from multiple etiologies of keratitis, we produced an *Acanthamoeba*-specific polyclonal peptide antibody against PBP of *A. castellanii* and evaluated its diagnostic potential.

Human corneal epithelial (HCE) cells (ATCC PCS-700–010) were cultured at 37°C with 5% CO₂ atmosphere in endothelial cell growth medium kits (KGM BulletKit) (Lonza, Portsmouth, New Hampshire, USA). *A. castellanii* (ATCC 30868) trophozoites were cultured in Peptone-Yeast-Glucose (PYG) media at 25°C with cysts being induced in encystment media at 25°C. *A. polyphaga*, *A. hatchetii*, *A. culbertsoni*, *A. royreba*, and *A. healyi* were kindly provided by Prof. Ho-Joon Shin (Ajou University, Suwon, Korea). *Fusarium solani* (NCCP 32678) was cultured in Sabouraud Dextrose (SD) media at 25°C, while *Pseudomonas aeruginosa* (NCCP 15920) were cultured in Brain Heart Infusion (BHI) media at 37°C.

The PBP of *A. castellanii* consists of 1,761 bp and encodes 586 amino acids with a calculated mass of 64.46 kDa (GeneBank accession No. MW683235.1). To design a peptide antigen with optimal antigenicity, amino acid sequences of PBP of *A. castellanii* were compared with that of *F. albosuccineum* (Fa_PBP, KAF4442825.1), *S. aureus* (Sa_PBP, BBA23260.1), and *P. aeruginosa* (Pa_PBP, KJI10303.1) (Fig. 1A). Amino acid sequence homology results revealed that PBP of *A. castellanii* had 21.5%, 20.3%, and 26.4% similarity with that of *F. albosuccineum*, *S. aureus*, and *P. aeruginosa*, respectively. The amino acids in the boxed area in Fig. 1A were selected for peptide antibody production using the peptide prediction software (AbFRONTIER, Seoul, Korea). We used RoseTTAFold to generate a 3-dimen-
sional model for conserved domains from the protein [20]. The RoseTTAFold predicted the entire structure of the PBP, and the amino acid sequence corresponding to the epitope portion is highlighted in red (Fig. 1B). Based on this illustration, we speculate that this antigenic site would serve as a good antigenic epitope as it is easily exposed to the outer surface.

The peptide sequence of PBP (C-EGDNKRSDVRELLRPADSD) used as the immunogen and the antibody raised against the peptide were purchased from AbFRONTIER [13]. To investigate the specificity of the PBP antibody, western blotting was conducted using 20 µg of HCE cells, A. castellanii, F. solani, S. aureus, and P. aeruginosa lysates. Cell lysates were resolved on 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk in Tris buffered saline containing 0.05% Tween 20 (TBST) for 2 h and incubated overnight at 4°C with the PBP antibody (1:1,000 dilutions in 5% skim milk). The membrane was incubated with HRP-conjugated anti-rabbit IgG (Sigma-Aldrich, St. Louis, Missouri, USA) (1:5,000 dilutions) for 1 h at room temperature (RT). Reactive bands were developed using Clarity Enhanced Chemiluminescence reagent (Thermo Fisher, Waltham, Massachusetts, USA). As shown in Fig. 2, the PBP antibody showed a strong reactive signal with A. castellanii, while immunoreactions were not observed with HCE cells and other causative agents of keratitis.

To confirm the specificity of the PBP antibody, immunocy-
Table 1. Specific detection of five reference Acanthamoeba spp. by immunocytochemistry assay

| Acanthamoeba spp. | Morphological types | Amoebic disease | Immunocytochemistry |
|-------------------|---------------------|-----------------|---------------------|
| A. polyphaga      | Group II            | AK              | ±                   |
| A. hatchetti      | Group II            | AK              | ±                   |
| A. culbertsoni    | Group III           | AK, GAE         | +                   |
| A. royreba       | Group III           | AK              | +                   |
| A. healyi        | Group III           | GAE             | +                   |

To verify the cross-reactivity of the PBP antibody, ICC assay was performed using A. castellanii trophozoites and cysts co-cultured with HCE cells. HCE cells (3 × 10^5 cells) were cultured on sterile cover glass in a 6-well plate. The following day, they were co-cultured with Acanthamoeba trophozoites (5 × 10^5 cells) and cysts (5 × 10^5 cells) for 5 h at 37°C in a 5% CO_2 incubator. The cells were fixed with 100% methanol for 5 min and subsequently permeabilized with PBST for 10 min at RT. The cells were subsequently blocked using blocking buffer (1% bovine serum albumin and 22.52 mg/ml glycine in PBST) for 30 min at RT. The cells were incubated overnight at 4°C with 1:200 diluted PBP antibody in blocking buffer and probed with CFL-488 fluorophore-conjugated antirabbit IgG antibody (1:400 dilutions) (Sigma-Aldrich) for 1 h at RT. After washing, cells were stained with VECTASHIELD mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) (Abcam, Burlingame, California, USA) and observed under a fluorescent microscope (Leica DMi8, Wetzlar, Germany). A. castellanii trophozoites (Fig. 3A) and cysts (Fig. 3B) showed strong immunoreactive signal with the PBP antibody (green). However, HCE cells did not show any reactive signal with the PBP antibody while the HCE cell nuclei were counterstained with DAPI (Fig. 3A, B). Additionally, a specific reaction of PBP antibody for A. castellanii was observed from the co-cultured cells of F. solani, S. aureus, and P. aeruginosa. HCE cells and trophozoites of A. castellanii were co-cultured with F. solani, S. aureus, and P. aeruginosa for 1 h. PBP antibody did not react with F. solani, S. aureus, P. aeruginosa, and HCE cells, whereas strong interaction of the PBP antibody was observed only with A. castellanii (Fig. 3C). These results demonstrated that the PBP antibody of A. castellanii specifically detected the A. castellanii trophozoites and cysts, which showed the potential for differential diagnosis for AK in mixed infection states.

To verify the cross-reactivity of the PBP antibody, ICC assay was performed using 5 different species of Acanthamoeba belonging to the morphological group II (A. polyphaga and A. hatchetti) and III (A. culbertsoni, A. royreba, and A. healyi). The trophozoites and cysts of the 5 Acanthamoeba species were detected by PBP antibody. However, A. polyphaga showed a weak reaction with the PBP antibody (Table 1).

Up to date, over 20 unique species of Acanthamoeba have been identified and 8 of them, which included A. castellanii, A. polyphaga, A. royreba, A. culbertsoni, A. hatchetti, A. griffin, A. quina, and A. lugdunensis have been reported to cause keratitis [21]. In this study, the PBP peptide antibody detected trophozoites and cysts of 6 Acanthamoeba species associated with AK (Fig. 3; Table 1). Interestingly, the PBP antibody was able to detect A. healyi (Table 1) that can cause GAE [22]. These results showed that the PBP antibody could detect several species of Acanthamoeba trophozoites and cysts causing keratitis and GAE.

In conclusion, our study demonstrated the ability of the PBP polyclonal peptide antibody of A. castellanii to recognize various species of Acanthamoeba, as well as the potential for differential diagnosis of AK. Further validation of findings presented here using human clinical samples is warranted. The PBP antibody may enhance sensitivity of antibody-based diagnostic methods for Acanthamoeba-associated diseases.

ACKNOWLEDGMENT

This work was supported by the National Research Foundation of Korea (NRF) grant funded by Korea government (MIST) (No. 2020R1A2C1005345).

CONFLICT OF INTEREST

The authors declare no conflict of interest related to this study.

REFERENCES

1. Marciano-Cabral F, Cabral G. Acanthamoeba spp. as agents of disease in humans. Clin Microbiol Rev 2003; 16: 273-307. https://doi.org/10.1128/CMR.16.2.273-307.2003
2. Schuster FL, Visvesvara GS. Free-living amoebae as opportunistic and non-opportunistic pathogens of humans and animals. Int J Parasitol 2004; 34: 1001-1027. https://doi.org/10.1016/j.ijpara. 2004.06.004
3. Page MA, Mathers WD. Acanthamoeba keratitis: a 12-year experience covering a wide spectrum of presentations, diagnoses, and outcomes. J Ophthalmol 2013; 2013: 670242. https://doi.org/10.1155/2013/670242
4. Fanselow N, Sirajuddin N, Yin XT, Huang AJW, Stuart PM. Acan-
13. Lee HA, Chu KB, Kim MJ, Quan FS, Kong HH, Moon EK. Chorismate mutase peptide antibody enables specific detection of *Acanthamoeba*. PLoS One 2020; 16: e0250342. https://doi.org/10.1371/journal.pone.0250342

14. Singh A, Sahu SK, Sharma S, Das S. *Acanthamoeba* keratitis versus mixed *acanthamoeba* and bacterial keratitis: comparison of clinical and microbiological profiles. Comea 2020; 39: 1112-1116. https://doi.org/10.1097/ICO.0000000000002337

15. Raghavan A, Baidwal S, Venkatapathy N, Ramplohan R. The *Acanthamoeba*-fungal keratitis study. Am J Ophthalmol 2019; 201: 31-36. https://doi.org/10.1016/j.ajo.2019.01.024

16. Moon EK, Choi HS, Park SM, Kong HH, Quan FS. Comparison of proteins secreted into extracellular space of pathogenic and non-pathogenic *Acanthamoeba* castellanii. Korean J Parasitol 2018; 56: 553-558. https://doi.org/10.3347/kjp.2018.56.5.553

17. Ko W, Kim S, Lee HS. Engineering a periplasmic binding protein for amino acid sensors with improved binding properties. Org Biomol Chem 2017; 15: 8761-8769. https://doi.org/10.1039/C7OB02165H

18. Edwards KA, Baemner AJ. Periplasmic binding protein-based detection of maltose using liposomes: a new class of biorecognition elements in competitive assays. Anal Chem 2013; 85: 2770-2778. https://doi.org/10.1021/ac303258n

19. Edwards KA, Randall EA, Tu-Maung N, Sannino DR, Feder S, Angert ER, Krafft CE. Periplasmic binding protein-based magnetic isolation and detection of thiamine in complex biological matrices. Talanta 2019; 205: 120168. https://doi.org/10.1016/j.talanta.2019.120168

20. Baek M, DiMaio E, Anishchenko I, Dauparas J, Ovchinnikov S, Lee GR, Wang J, Cong Q, Kinch LN, Schaeffer RD, Millán C, Park H, Adams C, Glassman CR, DeGiovanni A, Pereira JH, Rodrígues AV, van Dijk AA, Ebrecht AC, Opperman DJ, Sagmeister T, Buhlheller C, Pavkov-Keller T, Rathinaswamy MK, Dalwadi U, Yip CK, Burke JE, Garcia KC, Grishin NV, Adams PD, Read RJ, Baker D. Accurate prediction of protein structures and interactions using a three-track neural network. Science 2021; 373: 871-876. https://doi.org/10.1126/science.abj8754

21. Maycock NJ, Jayaswal R. Update on *Acanthamoeba* keratitis: diagnosis, treatment, and outcomes. Comea 2016; 35: 713-720. https://doi.org/10.1097/ICO.0000000000000804

22. Moon EK, Xuan YH, Kong HH. Microarray and KOG analysis of *Acanthamoeba* healyi genes up-regulated by mouse-brain passage. Exp Parasitol 2014; 143: 69-73. https://doi.org/10.1016/j.exppara.2014.05.012
