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PNA FISH assay uncovers *Enterococcus faecium* as a facultative intracellular bacterium in co-culture with *Acanthamoeba castellanii*

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Abstract

Enterococci species are gram-positive bacteria that cause different infections among hospitalized patients including urinary tract infections, bacteremia, intra-abdominal infections, and endocarditis. Acanthamoeba castellanii is an aquatic environmental eukaryote that is considered as an ideal cell in studying eukaryote-prokaryote interaction and as a powerful tool for the culture of some intracellular bacteria. We aimed to study interaction between Enterococcus faecium and A. castellanii in co-cultivation assay. The interaction has studied utilizing co-cultivation assay, Peptide nucleic acid technology and Fluorescence in situ hybridization combination. The utilised methods have uncovered an intracellular localisation of E. faecium inside A. castellanii. Growth and viability of the both interacted microorganisms are not inhibited snice both microorganisms have exhibited fluorescence as a detectable markers. Such this relation between the interacted microorganisms is defined symbiosis and A. castellanii as a host for the endosymbiont E. faecium.

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The fluorescent bacteria found outside and inside A. castellanii cells meaning that E. faecium is a facultative intracellular bacterium.

In conclusion, the facultative intracellular behaviour of E. faecium has shown for the first time in this study. We suggest that this behavior may be taken in considerations related to treatment and vaccination strategies against E. faecium.

Key words: *E. faecium*; facultative intracellular; *A. castellanii*; PNA FISH

INTRODUCTION

Bacteria are prokaryotic cells that can be subdivided to extracellular and intracellular microorganisms. Bacteria that multiply only inside or outside macrophage called intracellular or extracellular bacteria. While the bacteria that multiply inside and outside macrophage called facultative intracellular bacteria (1).

The bacterial genus *Enterococcus* includes over 50 species of gram-positive facultative anaerobes bacteria that exist in pairs or short chains. They grow optimally at 35 °C and can be found in diverse environments, from the soil to the gastrointestinal tract of animals and humans to the hospital environment. The first member of this genus was isolated in 1899 from a lethal case of endocarditis (reviewed in 2). Enterococci infections occurring mainly among hospitalized patients include urinary tract infections, bacteremia, intra-abdominal infections, and endocarditis, are caused by *Enterococcus faecalis* and *E. faecium* (2).

Acanthamoeba castellanii is an environmental eukaryote found worldwide in soil and fresh or salt water. Its life cycle has a reproductive trophozoite and a dormant cyst.

A. castellanii is an amphizoic protozoon that is able to be free-living amoeba (FLA) in environment or as parasite in humans and animals. It has an increased role as human pathogen causing encephalitis in the nervous system or keratitis in the eyes.

A. castellanii is characterised by long life because of encystation as well as excystation, phagocytosis and autofluorescence. *Acanthamoeba* cells emit green, red and blue fluorescent colors according to utilizing blue-, green-, and triple filter, respectively. It

resists antibacterial drugs and acts as predator or host to different bacteria. These characteristics make *Acanthamoeba* an ideal cell in studying eukaryote-prokaryote interaction and as a powerful tool for the culture of some intracellular bacteria (1, 3).

Live microorganisms produce an abundance of ribosomal RNAs that contain regions of highly conserved, species-specific sequences and are therefore ideal targets for identification assays such as Fluorescence in situ hybridization (FISH). However, the target sequences are frequently located in highly structured regions of the rRNA which are virtually inaccessible to DNA probes. The unique properties of peptic nucleic acid (PNA) probes allow access to these regions under conditions optimal for FISH resulting in a simple yet highly sensitive and specific hybridization assay (PNA FISH) suited for rapid and accurate identification of microorganisms. A drop from the concentrated faeces sample is fixed onto a microscope slide. PNA probe is added and hybridizes to the rRNA within the target microorganisms. Excess probe is removed during a stringent wash step and the slides are visualized using fluorescence microscopy. Fluorescing cells identify the target microorganism while non-florescence indicates the presence of a different microorganism in the sample (4).

Interaction of Enterococci with the free-living nematode *Caenorhabditis elegans* showed that *Enterococci* proliferated in and caused distention of the *C. elegans* intestine, but did not invade intracellularly or lysed intestinal cells (5).

In our this project we aimed to study interaction between *E. faecium* and *A. castellanii* cultivated together in ATCC medium no. 712 (ATCC) utilizing PNA FISH assay and fluorescence microscope.

MATERIAL AND METHODS

E. faecium isolate is obtained from Centre for Microbiological Preparedness, Swedish Institute for Infectious Disease Control, Sweden and *A. castellanii* (ATCC 30234) from the American Type Culture Collection, Manassas, Virginia, USA.

Co-cultivation of *E. faecium* and *A. castellanii* prepared as described before (1). Briefly, *A. castellanii* was grown at 30 ^{O}C to a final concentration of 10^6 cells /mL in ATCC medium no. 712. *E. faecium* was grown on blood agar plate for overnight at 37 ^{O}C and few

bacterial colonies were regrown in LB broth to an OD_{600} of 0.6. Co-culture of each *E. faecium* and *A. castellanii* was incubated in 75 cm^2 cell culture flasks (Corning Costar) filled with 30 ml ATCC medium no. 712 containing an initial concentration of 10^5 cells *A. castellanii* per mL and 10^6 cells *E. faecium* per ml. The flasks were incubated without shaking at 30 ^oC at the Centre for Microbiological Preparedness, Sweden. After two days co-cultivation, samples were prepared according to AdvanDx assay (6). Briefly, the samples were preceded for fixation, PNA probe hybridization, washing of unbound and excess PNA probe, and examined by fluorescence microscope at AdvanDx laboratory, AdvanDx A/S, Bygstubben 11, 2950 Vedbæk, Denmark.

RESULT

Growth and viability of *E. faecium* and *A. castellanii*

After two days co-cultivation of *E. faecium* with *A. castellanii*, samples were prepared according to AdvanDx assay for fluorescence microscopy. The results showed that *E. faecium* grew together with *A. castellanii* in co-cultivation flask and viability of *E. faecium* was not affected since it emitted the designed fluorescence by AdvanDx as a detectable marker that was not quenched *A. castellanii* autofluorescence.

In the other hand, *A. castellanii* cells could attach and took up *E. faecium* cells as well as both acanthamoebae and uptaken bacterial cells were still viable and detectable by the used fluorescence microscopy as shown in figures 1-3.

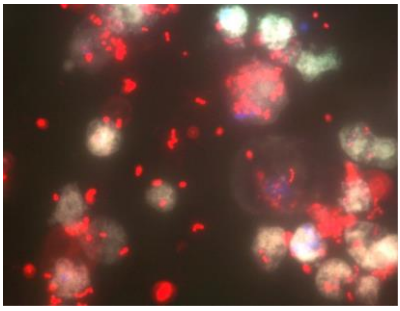

Figure 1: Red fluorescent *E. faecium* localised outside and inside *A. castellanii* cells that emitted blue autofluorescence through the triple filter. Micrograph magnification was1000x

E. faecium localised outside and inside *A. castellanii*

Notably, *E. faecium* found inside 60%, 70% and 50% of *A. castellanii* cells (Figures 1-3) in addition many cells of *A. castellanii* were overwhelmed by the intracellular *E. faecium* as shown in figures 1 and 2.

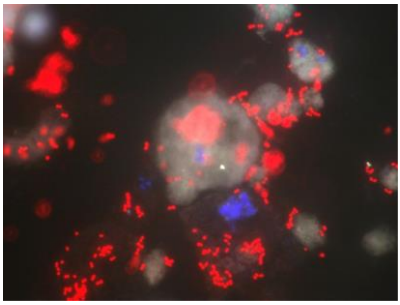

Figure 2: Red fluorescent *E. faecium* localized outside and inside *A. castellanii* cells that emitted blue autofluorescence through the triple filter. Micrograph magnification was1000x.

Stability and detection ability of fluorescent-labeled PNA probes

Different fluorescent probes were able to detect *E. faecium* whether it localised outside or inside *A. castellanii* (figures 1-3). Furthermore, *E. faecium* that considered extracellular bacterium (5) (Yuen GJ, Ausubel FM 2018) was localised with detectable and stable fluorescence outside and inside the environmental macrophage *A. castellanii*. However, utilising green fluorescent-labeled PNA probes such as uniprobe GNS we found that *E. faecium* emitted green fluorescence and *A. castellanii* red autofluorescence through the green filter as shown in figure 3.

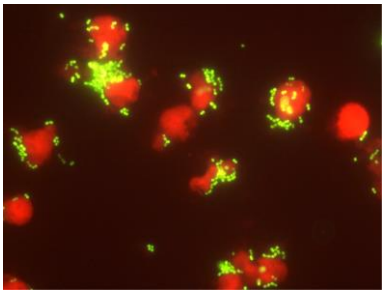

Figure 3: Green fluorescent *E. faecium* localised outside and inside *A. castellanii* cells that emitted red autofluorescence through the green filter. Micrograph magnification was 500x.

DISCUSSION

Surprisingly, *E. faecium* that considered previously as an extracellular bacterium (5), it interacted as facultative intracellular bacterium in our current study since it localised outside and inside *A. castellanii* as shown in figures 1-3. This finding agreed with *E. faecalis* that described previously as a facultative intracellular bacterium (7). In this context, Yuen and Ausubel 2014 studied interaction of Enterococci with *C. elegans* and found that *E. faecalis* distended intestine of *C. elegans*, induced intestinal stasis, and later killed the worm while *E. faecium* simply distended the intestine of *C. elegans* without killing (5). Interestingly, they observed that the maximal lifespan of *C. elegans* fed live *E. faecium* was considerably longer that the lifespan of worms fed heat-killed *E. faecalis* (5) uncovering that the live wild *E. faecium* is likely adapted to coexist with the free-living worm *C. elegans*. In line with these findings, interaction of the facultative intracellular bacterium *Vibrio cholerae* with free-living amoeba *A. castellanii* showed that outer membrane protein A (*OmpA*) mutant of *V. cholerae* survived longer than wild-type *V. cholerae* when cultivated alone, compared to co-cultivation with *A. castellanii* that enhanced the survival of both bacterial strains. Moreover, the co-cultivation of the *OmpA* mutant of *V. cholerae* decreased the viability of *A. castellanii* since this bacterial strain released more OMVs than wild-type *V. cholerae* (8).This significant production of OMVs might have enriched the cultivation medium and supported longer survival of the mutant strain compared with the wild-type *V. cholerae* (8). Thus the OMVs might act as a virulence factors when they supported a long survival of the bacterium and decreased viability of the interacted amoebae. However, *V. cholerae* and *A. castellanii* interaction uncovered that *V. cholerae* behaved as facultative intracellular bacteria and adapted to survive better in association with eukaryotes (8-12). In our current article, *E. faecium* interacted as facultative intracellular bacterium since it localised inside *A. castellanii* and found in the cultivation medium as shown by electron microscopy.

Before AdvanDx PNA FISH assay, extracellular and intracellular bacteria can not be identified, differentiated and visualised by fluorescent microscopy unless the bacteria are cloned by a plasmid carrying green fluorescent protein (GFP) and treated with

R-phycoerythrin-labeled antibodies specific for the bacteria (13). Astonishingly, AdvanDx PNA FISH assay identifies the bacteria whether they are intracellularly or extracellularly located as shown in this study. While plasmid cloning and antibody-labeling are two separated methods that do not utilise for detection but only for visualisation of well-known bacteria.

CONCLUSIONS

This is the first article describing an intracellular localisation of *E. faecium* in an environmental macrophage *A. castellanii* that can be considered as an ecological reservoir of Enterococci bacteria and may point out *E. faecium* as a facultative intracellular bacterium.

This facultative intracellular behaviour of *E. faecium* in this study taken together with the behaviour of *E. faecalis* that shown before, may be taken in considerations related to treatment, vaccination strategies and diagnosis of these bacteria according to their extracellular- and intracellular existence.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

REFERENCES

1. Abd H. Interaction between waterborne pathogenic bacteria and *Acanthamoeba castellanii*, Doctoral thesis 2006, ISSN/ISBN, 91-7140 - 569 - 0. Karolinska Institute, Stockholm, Sweden www.diss.kib.ki.se
2. Dubin K, Pamer EG. Enterococci and their interactions with the intestinal microbiome. Microbiol Spectr. 2014; 5 (6):10.1128/microbiolspec.BAD-0014-2016.

3. Abd H, Saeed A, Alharbi S, Alshamari F. Diagnosis of Keratitis by Cultivation, Fluorescence Microscopy and PCR has uncovered that Acanthamoeba Emits Auto Fluorescence. SOJ Microbiol & Infect Dis.2017; 5: 1-5.
4. Stender H. PNA FISH: an intelligent stain for rapid diagnosis of infectious diseases. Expert Review of Molecular Diagnostics. 2003; 3:649- 655.
5. Yuen GJ, Ausubel FM. Enterococcus infection biology: lessons from invertebrate host models. J Microbiol. 2014; 52: 00-210. doi:10.1007/s12275-014-4011-6.
6. AdvanDx PNA FISH Rapid identification of bacteria and yeast directly from positive blood cultures. http://www.opgen.com/advandx-pathogen-id/advandx-pna-fish/
7. Gentry-Weeks CR, Karkhoff-Schweizer R, Pikis A , Estay M, JM. Keith JM. Survival of *Enterococcus faecalis* in mouse peritoneal macrophages. Infect. Immune. 1999; 67: 2160 - 2165.
8. Valeru SP, Shanan S, Alossimi H, Saeed A, Sandström G, Abd H. Lack of outer membrane protein A enhances the release of outer membrane vesicles and survival of Vibrio cholerae and suppresses viability of *Acanthamoeba castellanii*. Int. J. Microbiol. 2014; ID: 610190. http://dx.doi.org/10.1155/2014/610190
9. Shanan S, Bayoumi M, Saeed A, Sandström G, Abd H. Swedish isolates of *Vibrio cholerae* enhance their survival when interacted intracellularly with *Acanthamoeba castellanii*. Infection Ecology and Epidemiology. 2016; 6: 31060
10. Abd H, Shanan S, Saeed A, Sandström G. Survival of Vibrio cholerae inside Acanthamoeba and detection of both microorganisms from natural water samples may point out the Amoeba as a protozoal host for V. cholerae. Review. J Bacteriol Parasitol. 2001; S1-003. doi:10.4172/2155- 9597
11. Abd H, Saeed A, Weintraub A, Sandström, G. Vibrio cholerae O139 requires neither capsule nor LPS O side chain to grow inside Acanthamoeba castellanii. J Med Microbiol. 2009; 58: 125–131

12. Abd H, Saeed A, Weintraub A, Nair GB, Sandström G. Vibrio cholerae O1 strains are facultative intracellular bacteria, able to survive and multiply symbiotically inside the aquatic free-living amoeba Acanthamoeba castellanii. FEMS Microbiology Ecology. 2007; 60: 33-9.
13. Abd H, Johansson T, Igor G, Sandström G, Forsman M. Survival and growth of *Francisella tularensis* in *Acanthamoeba castellanii*. Applied & Environ Microbiol. 2003; 69: 6006 - 6