Technische Universität Braunschweig

Zoologisches Institut

TU Braunschweig, Zoological Institute • D-38106 Braunschweig

To the Editor of the
Journal of Visualized Experiments
1 Alewife Center Suite 200
Cambridge, MA 02140
USA

Prof. Dr. Reinhard W. Köster
TU Braunschweig, Zoological Institute
Cellular and Molecular Neurobiology
Spielmannstr. 7, D-38106 Braunschweig

Phone: 0531/391 3230
Fax: 0531/391 3222
E-Mail: r.koester@tu-bs.de
Homepage http://www.zoologie.tu-bs.de/index.php/en/cellular-molecular-neurobiology
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Dear Editor,

please find enclosed our manuscript entitled “Development of zebrafish infection model for *Clostridoides difficile*” which we would like to submit for publication in the Journal of Visualized Experiments.

Infections with *Clostridium difficile* are among the most common hospital acquired infections world-wide and account for numerous death per year, because in the recent years multi-resistant strains for this infectious pathogen have emerged and are spread around the world. Yet the response of the innate immune system to *Clostridioides difficile* infections (CDI) are understudied, because infections of the intestine occur deep inside the body and innate immune cells are highly motile. Thus a small, nearly transparent vertebrate would serve as a well-suited infection model to allow for monitoring pathogen as well as innate immune cell behaviour directly *in vivo* by high resolution microscopy. Zebrafish larvae contain these advances and are also molecularly tractable to generate transgenic strains with fluorescently labeled macrophages or neutrophils. However, currently available zebrafish reporter strains only display weak fluorescence in innate immune cells. In addition, while protocols for infection of zebrafish with bacteria have been established these are usually suited for aerobic but not anaerobic pathogens such as *Clostridium difficile*.

We have therefore set out to establish a fluorescent labelling method for this Gram-positive pathogen. Furthermore, we provide methods for different infection routes for this bacterial strain either by microinjection or by microgavage through the open mouth of zebrafish larvae to deposit the pathogen directly inside the gastro-intestinal tract. In addition, we provide experimental approaches for confirming successful infection of the larval zebrafish intestinal tract. For this we demonstrate protocols for recovering administered bacteria either by direct culture or by culturing bacteria derived from spore formation. Furthermore, we demonstrate differences in pathogen survival when gnotobiotic zebrafish are used compared to wild type larvae. In summary, we provide a package of methods suited to investigate bacterial infections of anaerobic pathogens and resulting innate immune cell response behavior in transparent zebrafish larvae. We are convinced that these technical advancements will be of help for infection biologists and zebrafish researchers alike and therefore hope for your positive approval for publishing this manuscript in the Journal of Visualized Experiments.

Yours sincerely,

R. Köster

(Reinhard Köster)