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Lightning up the worm: How to probe fungal virulence in an alternative mini-host by bioluminescence

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Infections caused by fungal pathogens pose a serious and steadily increasing threat to susceptible individuals worldwide[1] with a specific impact on treatment of immunocompromised patients. Fungi represent a distinct kingdom among the Eukarya, making the design and validation of therapeutic compounds that may counteract fungal infections a highly challenging and unfortunately massively neglected field in the pharmaceutical pipeline.[2] Mechanisms and determinants of fungal pathogenesis are far from being understood comprehensively but this is a pre-requisite to define what has been coined the human virulome of pathogenic fungi.[3] As an infectious disease is commonly the outcome of a potential pathogen encountering a susceptible host, both sides of this intertwined interplay need to be studied with the aim to put fungal characteristics in the appropriate context of virulence.[4] Therefore, suitable models of infection are an essential requirement when testing distinct features of the pathogen as virulence-determining factors or to validate therapeutic interventions such as efficiencies of antimycotic substances. Small vertebrates like mice, rats, rabbits, guinea pigs, or hamsters have been established extensively for such purposes, based on their relative convenience with respect to handling and manipulation. The in-depth knowledge of the murine immune system that is accompanied by its highly advanced molecular biology and genetic accessibility has cemented the prime role of mice as hosts in infection studies, serving as proxy for the susceptible human host that is confronted with fungal pathogens. Yet, financial, infrastructural, and especially ethical issues limit the implementation of mammalian infection models to study the virulome of fungi. Apart from this, following the course of infection in individual hosts to assess fungal burden, histopathology, or immune responses is generally hampered by the application of invasive procedures that usually require culling of the infected cohort animal-by-animal.

Addressing these aspects, Coste and coworkers describe in the recent issue of *Virulence* their efforts to combine 2 of the most recent developments in fungal infection research, that is the use of an alternative insect mini-host accompanied by the implementation of a suitable reporter system based on bioluminescence that allows longitudinal studies in single infected animals.[5] Driven by the need for alternatives replacing mammalian systems to study fungal infections, established model organisms such as the zebrafish *Danio rerio*, the nematode *Ceanorhabditis elegans* or the soil amoeba *Dictyostelium discoideum* and *Acanthamoeba castellanii* have been validated in recent years to cover various characteristics of fungal virulence and host response.[6-12] This has been complemented by making use of embryonated chicken eggs, which allowed virulence studies addressing relevant aspects such as invasion, dissemination, or immune reactions.[13] In promoting such initiatives, invertebrate insect hosts have a longstanding tradition to elucidate mechanisms of immunity against fungal pathogens and virulence determinants, ranging from seminal early studies in the fruit fly *Drosophila melanogaster* that opened the field of receptor-mediated recognition of pathogen-associated molecular patterns, the so-called PAMPs, to recent ones in locusts that appear suitable to mimic invasion of the central nervous systems by pathogenic fungi.[14-16] Due their ease of housing and handling and thereby obviating administrative as well as ethical concerns, insect larvae have emerged as most suitable for infection studies; this is accompanied by several other benefits, such as relatively low purchase costs, their survival at appropriate temperatures that support growth of fungal pathogens, or the feasibility of precise and non-traumatic inoculation with defined infectious doses due to their relatively large size.

For the opportunistic pathogen *Candida albicans*, the silkworm *Bombyx mori* or caterpillars of the tobacco hornworm *Manduca sexta* had been established most

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recently as suitable replacement hosts to monitor virulence traits, while larvae of the greater wax moth *Galleria mellonella* have served as predominant mini-host for *Candida* infections since the year 2000.[17-19] These larvae lack an adaptive immune response but hold various hemocytes that eliminate invading pathogens *via* phagocytosis; moreover, humoral components of innate immunity, like antimicrobial peptides, are mounted upon infection, with differences being evident for the response against bacterial or fungal pathogens.[20] Wax moth larvae have served to address various aspects of the host-fungus interplay in the context of pathogenesis, ranging from pattern recognition triggering nodulation to filamentation and other traits of virulence and even the efficiency of antifungals.[21-23] A systematic analysis of *C. albicans* mutants impaired in the yeast-to-hyphal transition in both the murine systemic infection model as well as the *Galleria* system revealed a reliably good correlation,[22] and studies with the yeast pathogen *Cryptococcus neoformans* or the human-pathogenic mold *Aspergillus fumigatus* have led to similar conclusions.[24,25] A recent study from the Coste group however also demonstrated significant discrepancies between the 2 infection models.[26] Yet, *G.* mellonella larvae have earned their merits in studying fungal infections that is only dampened by the evident lack of a reference genome sequence and missing tools of molecular biology for genetic engineering.

When following the course of disease and/or therapeutic treatment in an appropriate infection model system, crucial parameters such as symptomatic signs but also pathogen distribution and load need to be monitored. In this respect, sensitive reporter systems have been developed that are based on bioluminescence, i.e. photon emission conjunct with substrate oxidation that is catalyzed by light-generating enzymes. These so-called luciferases have evolved in prokaryotes as well as eukaryotes, and several enzymes of either origin are nowadays established, differing with respect to physico-chemical characteristics such as the nature of (co-)substrates, signal peak wavelength, or emission kinetics.[27] Emitted photons are commonly detected by the use of highly sensitive charged coupled device (CCD) cameras to yield a quantitative read-out of bioluminescence. Accordingly, a longitudinal insight about pathogen distribution in the infected organism might be gained without the need for terminal inspection, which evidently reduces the number of hosts to be monitored. For fungal pathogens, several studies have demonstrated the usefulness of but also the limitations of bioluminescence imaging in murine infection systems.[28-35] A major restriction lies in the obligate need for external substrate application, which is in contrast to bacterial pathogens that might be transformed with thoroughly characterized and adapted bioluminescence operons.[36] Due to the orthogonality of such systems, these cannot be employed in fungal organisms, while eukaryotic bioluminescent systems are only characterized with respect to the luciferase activity but not substrate-generating pathways.

In the recent issue of *Virulence*, Eric Delarze and colleagues have described the fruitful combination of both approaches, employing *Galleria mellonella* larvae as hosts for infections by the human commensal *Candida albicans* and implementing bioluminescence as reporter read-out to enhance studies on virulence traits as well as antifungal treatment.[5] By transferring a validated and optimized expression module for surface display of the luciferase enzyme from the copepod *Gaussia princeps*[29] to a wild-type isolate and congenic deletion strains, bioluminescent signals could be quantified *ex vivo* from the pulp of infected and sacrificed larvae that correlated to fungal burdens deduced from colony forming units to some degree. Most importantly, the fungal infection could also be monitored over time in living wax moth larvae by using a non-toxic and water-soluble formulation of the substrate coelenterazine, WCTZ. This allowed *in vivo* kinetic studies of *C. albicans* infections in this alternative host and represents a step forward in elucidating virulence characteristics of this common yeast pathogen and in monitoring options for antifungal treatment. From this study, further perspectives emerge but also shortcomings and limitations of the system became evident: Obviously the described achievements pave the road for large scale studies to yield significant insights at high reliability without the need for infecting numerous cohorts of susceptible mice. This proof-of-concept study might also spark additional initiatives with other fungal pathogens for which bioluminescence as well as the wax moth larvae infection model had been successfully established. Yet, drawbacks emerge from the apparent lack of a standardized set of congenic *C. albicans* strains that carry the integrated reporter constructs at defined and identical numbers. Infecting wax moth larvae can also not reflect the various facets of *C. albicans* pathogenicity that range from superficial colonization to systemic dissemination. In this context, however, the *Gaussia*-derived bioluminescence system had been characterized exhaustingly in murine infection models to reveal shortcomings that may be associated with substrate instability, emission characteristics, or tissue penetration issues.[29] Accordingly, the necessity of external substrate application in bioluminescence studies on fungal pathogens is still an unresolved issue that needs to be addressed to significantly further the field. The recent insights by Delarze *et al*., however, make a strong case for developing bioluminescence imaging approaches in alternative mini-hosts with the aim to study fungal virulence.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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