Review

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Usefulness of silkworm as a host animal for understanding pathogenicity of *Cryptococcus neoformans*

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Summary **We propose *Cryptococcus neoformans* infection model using silkworm for understanding cryptococcosis and screening of therapeutically effective antibiotics. Silkworm is an insect whose rearing methods were established through a long history of the sericulture industry. Silkworm facilitates experiments using a large number of individuals because of low cost for rearing and few ethical problems caused by killing animals. Silkworm can be reared at 37˚C to perform infection experiments at same temperature to human body. Injection of accurate amounts of samples into hemolymph of silkworm by usual syringes is easy to be done since silkworm has an appropriate size to handle. Moreover two injection methods, injection into hemolymph and intestine, are distinguishable for silkworms. The former is correspondent to intravenous injection, and the latter is to oral administration in humans. Taking these advantages of silkworms as host animals, it is possible to evaluate the virulence factors in *C. neoformans* and the therapeutic efficacy of antifungal agents.**

Keywords: *Cryptococcus neoformans*, infectious disease, silkworm

1. Introduction

Cryptococcus neoformans is a pathogenic fungus that causes cryptococcosis in humans (*1*). *C. neoformans* is frequently isolated from immunocompromised patients. Cryptococcosis is one of the most causes of death in AIDS patients (*2*). Basic study using animal models that imitates human infectious disease is necessary to understand pathogenicity of *C. neoformans* and to establish prevention and therapeutic strategies against cryptococcosis. Various *C. neoformans* infection models with mammalian hosts have been proposed (*3-5*). Mammalian models, however, have problems of not only high cost but also of ethical issues from a view of animal welfare. Therefore, establishment of invertebrate animals for searching virulence factors of *C. neoformans* and for screening of therapeutic agents is desired. Invertebrate animals have advantages compared to mammals: *1)* low cost for rearing, *2)* smaller space needed for rearing, *3)* less ethical issues by killing animals, and *4)* less amount of samples because of smaller body size (Table 1). At present, besides silkworm (*Bombyx mori*) proposed by us (*6*), fruit fly (*Drosophila melanogaster*), nematode (*Caenorhabditis elegans*), and larvae of greater wax moth (*Galleria mellonella*) are proposed as host animals of *C. neoformans* infection (*7-9*). In this review, we describe usefulness of these invertebrates as host of *C. neoformans* infection.

2. *C. neoformans* infection model using silkworm

Silkworm is a larva of domesticated silkmoth, *Bombyx mori*. The rearing method is well-established during a long history of sericulture in Asian countries. We previously proposed various disease models, such as infectious diseases by pathogens, diabetes, and drug-induced tissue injury, and use of these systems for screening of drug candidates (*10-13*). Among them, infection models including fungal infection are highly effective for screening virulence factors of pathogens and therapeutically effective antibiotics (*14,15*). Silkworm fungal infection models were reported for four species of fungi, *Cryptococcus neoformans*, *Candida albicans*, *Candida grabrata*, and *Candida tropicalis* (*16*). Several

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Table 1. *In vivo* infection models of *Cryptococcus neoformans*

Items	Model animals	Cost for rearing	Space for rearing	Application to the ethics committee	Escape ability to require the biosafety	Time required for die after injection	Temperature after infection	Quantitative injection of samples by a syringe	Reported route of administration	Individual weight
Silkworm	*Bombyx mori*	Low	Small	Not necessary	Low	2-3 days	37°C	Easy	Intrahemolymph, intragut injections	1-2 g
Fruit fly	*Drosophila melanogaster*	Low	Small	Not necessary	High	3-4 days	25°C	Difficult	Intrahemolymph injection, oral administration	0.5-2 mg
Nematode	*Caenorhabditis elegans*	Low	Small	Not necessary	Low	2-25 days	25°C	Difficult	Oral administration	1 μg
Larvae of greater wax moth	*Galleria mellonea*	Low	Small	Not necessary	Low	4-20 days	30°C, 37°C	Easy	Intrahemolymph injection	250 mg
Mice	*Mus musculus*	High	Large	Necessary	High	6-40 days	37°C (body temperature)	Easy	Intratracheal, intravenous, intraperitoneal injection	15-40 g

ND: Note Determined.

fungal virulence factors were identified using these fungal models (*17,18*). We previously reported that silkworm *C. neoformans* infection model was useful for quantitative evaluation of *C. neoformans* pathogenecity and therapeutic effects of antifungal drugs (*6*). Silkworm survive at least three days at 37˚C, therefore, silkworm can be used for infection experiments at 37˚C (*19*). Injection of live fungal suspension of *C. neoformans* H99 strain into silkworm hemolymph causes killing effects at 37˚C. *C. neoformans* Serotype A, which has high pathogenicity against mammals, was shown to kill silkworm with less number of fungi than Serotype D, which has low pathogenicity against mammals. In other words, silkworm infection model can distinguish strains having different levels of pathogenicities.

Deletion mutants of *gpa1*, *pka1*, and *cna1* genes, which were reported to be needed to exhibit pathogenicity of *C. neoformans* against mammals (*20-22*), also showed higher LD_{50} values than that of parent strain. This means that these genes are also needed to exhibit pathogenicity against silkworms as well as mammals. Intra-hemolymph injections of amphotericin B, flucytosine, fluconazole, and ketoconazole showed therapeutic effect against death of silkworm by *C. neoformans* infection. On the other hand, amphotericin B, which is not absorbed from gut in mammals, did not show the therapeutic effect by intra-gut injection, which corresponds to oral administration in humans. This result can be explained by that amphotericin B is not absorbed from gut also in silkworm. From these results, we expect that silkworm *C. neoformans* infection model is useful as an alternative method to evaluate therapeutic efficacy of antifungal drugs.

3. *C. neoformans* infection models using other invertebrate models

D. melanogaster, a fruit fly, is widely used as a model animal (*23*). An advantage of *D. melanogaster* as an experimental animal is that different kinds of genetic approaches can be applicable (*24*). Using mutant libraries of *D. melanogaster*, the host immune system related to *C. neoformans* infection has been elucidated. In particular, mutants of Imd and Toll pathways, which are signal pathway related to innate immune system of *D. melanogaster*, were analyzed in the *C. neoformans* infection model (*8*). A mutant of Toll pathway was susceptible to *C. neoformans* infection, whereas a mutant of Imd pathway was not susceptible. Thus, Toll pathway plays a key role in the innate immunity against *C. neoformans*. Adult flies, not larvae, are generally used in infection experiments using *D. melanogaster*. Special micro injectors with glass syringes, not usual clinical syringe, are needed for injection, because the size of adults fly is very small, 2-3 mm. Therefore, determination of LD_{50} and ED_{50} by injection of precise volume of sample solution is very difficult. Experiments

with adults of *D. melanogaster* at 37˚C are not possible, since the flies cannot be reared at high temperatures.

C. elegans also provides excellent animal model to perform genetic studies (*25*). Genes related to innate immunity in *C. neoformans* infection were identified using *C. elegans* (*26-28*). Capsule and other virulence factors of *C. neoformans*, which are needed to exhibit the pathogenicity against mammals, were reported to be needed to exhibit the pathogenicity against *C. elegans* (*7*). *C. elegans* was also used to screen virulence factors of *C. neoformans* (*29-36*). Moreover, *C. neoformans* infection model with *C. elegans* was also used to evaluate therapeutic effects of antifungal reagents (*37,38*).

G. mellonella is large moth which belong to Lepidoptera, same as silkworm. *G. mellonella* has been studied as infection models of fungi including *C. neoformans* (*39-42*). *G. mellonella* is possible to perform infection experiments at 37˚C. Novel virulence factors of *C. neoformans* was also screened using the *G. mellonella* model (*43*). Evaluation of therapeutic effects of antifungal drugs was reported with an infection model of *C.neoformans* with *G. mellonella* (*9,44*). Since its big body size, *G. mellonella* has a capacity to collect a large volume of hemolymph similar to silkworm (*45*).

4. Conclusions

Invertebrate animal hosts, silkworm (*B. mori*), fruit fly (*D. melanogaster*), nematode (*C. elegans*), and larva of greater worm moth (*G. mellonella*), are expected to solve problems of high cost and ethical issues from a view of animal welfare in *C. neoformans* infection models using mammals, such as mice and rats. Silkworm has several advantages compared to *D. melanogaster* and *C. elegans*: *1)* bigger body size of individuals and lower motility, which facilitate quantitative injection of samples, *2)* survival at 37˚C, body temperature of human, and *3)* available for two types of injection ways, intra-hemolymph and intra-gut. Whereas, *D. melanogaster* and *C. elegans* have advantages of experimental systems for genetics, such transgenic techniques can be applicable for silkworms (*46-48*). Using these techniques, understanding of host immune system in silkworm responding to *C. neoformans* infection is important issue in future.

References

1. Morschhauser J. Regulation of multidrug resistance in pathogenic fungi. Fungal Genet Biol. 2010; 47:94-106.
2. Park BJ, Wannemuehler KA, Marston BJ, Govender N, Pappas PG, Chiller TM. Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. AIDS. 2009; 23:525-530.
3. Perfect JR, Lang SD, Durack DT. Chronic cryptococcal meningitis: a new experimental model in rabbits. Am J Pathol. 1980; 101:177-194.
4. Goldman D, Lee SC, Casadevall A. Pathogenesis of pulmonary *Cryptococcus neoformans* infection in the rat. Infect Immun. 1994; 62:4755-4761.
5. Zaragoza O, Alvarez M, Telzak A, Rivera J, Casadevall A. The relative susceptibility of mouse strains to pulmonary *Cryptococcus neoformans* infection is associated with pleiotropic differences in the immune response. Infect Immun. 2007; 75:2729-2739.
6. Matsumoto Y, Miyazaki S, Fukunaga DH, Shimizu K, Kawamoto S, Sekimizu K. Quantitative evaluation of cryptococcal pathogenesis and antifungal drugs using a silkworm infection model with *Cryptococcus neoformans*. Journal of Applied Microbiology. 2012; 112:138-146.
7. Mylonakis E, Ausubel FM, Perfect JR, Heitman J, Calderwood SB. Killing of *Caenorhabditis elegans* by *Cryptococcus neoformans* as a model of yeast pathogenesis. Proc Natl Acad Sci U S A. 2002; 99:15675-15680.
8. Apidianakis Y, Rahme LG, Heitman J, Ausubel FM, Calderwood SB, Mylonakis E. Challenge of *Drosophila melanogaster* with *Cryptococcus neoformans* and role of the innate immune response. Eukaryot Cell. 2004; 3:413-419.
9. Mylonakis E, Moreno R, El Khoury JB, Idnurm A, Heitman J, Calderwood SB, Ausubel FM, Diener A. *Galleria mellonella* as a model system to study *Cryptococcus neoformans* pathogenesis. Infect Immun. 2005; 73:3842-3850.
10. Matsumoto Y, Ishii M, Hayashi Y, Miyazaki S, Sugita T, Sumiya E, Sekimizu K. Diabetic silkworms for evaluation of therapeutically effective drugs against type II diabetes. Sci Rep. 2015; 5:10722.
11. Matsumoto Y, Sumiya E, Sugita T, Sekimizu K. An invertebrate hyperglycemic model for the identification of anti-diabetic drugs. PLoS One. 2011; 6:e18292.
12. Inagaki Y, Matsumoto Y, Kataoka K, Matsuhashi N, Sekimizu K. Evaluation of drug-induced tissue injury by measuring alanine aminotransferase (ALT) activity in silkworm hemolymph. BMC Pharmacol Toxicol. 2012; 13:13.
13. Kaito C, Akimitsu N, Watanabe H, Sekimizu K. Silkworm larvae as an animal model of bacterial infection pathogenic to humans. Microb Pathog. 2002; 32:183-190.
14. Kaito C, Kurokawa K, Matsumoto Y, Terao Y, Kawabata S, Hamada S, Sekimizu K. Silkworm pathogenic bacteria infection model for identification of novel virulence genes. Mol Microbiol. 2005; 56:934-944.
15. Hamamoto H, Urai M, Ishii K, *et al*. Lysocin E is a new antibiotic that targets menaquinone in the bacterial membrane. Nat Chem Biol. 2015; 11:127-133.
16. Ishii M, Matsumoto Y, Sekimizu K. Usefulness of silkworm as a model animal for understanding the molecular mechanisms of fungal pathogenicity. Drug Discov Ther. 2015; 9:234-237.
17. Hanaoka N, Takano Y, Shibuya K, Fugo H, Uehara Y, Niimi M. Identification of the putative protein phosphatase gene PTC1 as a virulence-related gene using a silkworm model of *Candida albicans* infection. Eukaryot Cell. 2008; 7:1640-1648.
18. Ueno K, Matsumoto Y, Uno J, Sasamoto K, Sekimizu K, Kinjo Y, Chibana H. Intestinal resident yeast *Candida glabrata* requires Cyb2p-mediated lactate assimilation to adapt in mouse intestine. PLoS One. 2011; 6:e24759.
19. Kaito C, Usui K, Kyuma T, Sekimizu K. Isolation of mammalian pathogenic bacteria using silkworms. Drug Discov Ther. 2011; 5:66-70.

20. Alspaugh JA, Perfect JR, Heitman J. *Cryptococcus neoformans* mating and virulence are regulated by the G-protein α subunit GPA1 and cAMP. Genes Dev. 1997; 11:3206-3217.
21. Odom A, Muir S, Lim E, Toffaletti DL, Perfect J, Heitman J. Calcineurin is required for virulence of *Cryptococcus neoformans*. EMBO J. 1997; 16:2576-2589.
22. D'Souza CA, Alspaugh JA, Yue C, Harashima T, Cox GM, Perfect JR, Heitman J. Cyclic AMP-dependent protein kinase controls virulence of the fungal pathogen *Cryptococcus neoformans*. Mol Cell Biol. 2001; 21:3179-3191.
23. Matthews KA, Kaufman TC, Gelbart WM. Research resources for Drosophila: the expanding universe. Nat Rev Genet. 2005; 6:179-193.
24. Venken KJT, Bellen HJ. Emerging technologies for gene manipulation in *Drosophila melanogaster*. Nat Rev Genet. 2005; 6:167-178.
25. Kaletta T, Hengartner MO. Finding function in novel targets: *C. elegans* as a model organism. Nat Rev Drug Discov. 2006; 5:387-398.
26. Kerry S, TeKippe M, Gaddis NC, Aballay A. GATA transcription factor required for immunity to bacterial and fungal pathogens. PLoS One. 2006; 1:e77.
27. TeKippe M, Aballay A. *C. elegans* germline-deficient mutants respond to pathogen infection using shared and distinct mechanisms. PLoS One. 2010; 5:e11777.
28. Means TK, Mylonakis E, Tampakakis E, Colvin RA, Seung E, Puckett L, Tai MF, Stewart CR, Pukkila-Worley R, Hickman SE, Moore KJ, Calderwood SB, Hacohen N, Luster AD, El Khoury J. Evolutionarily conserved recognition and innate immunity to fungal pathogens by the scavenger receptors SCARF1 and CD36. J Exp Med. 2009; 206:637-653.
29. Mylonakis E, Idnurm A, Moreno R, El Khoury J, Rottman JB, Ausubel FM, Heitman J, Calderwood SB. *Cryptococcus neoformans* Kin1 protein kinase homologue, identified through a *Caenorhabditis elegans* screen, promotes virulence in mammals. Mol Microbiol. 2004; 54:407-419.
30. Tang RJ, Breger J, Idnurm A, Gerik KJ, Lodge JK, Heitman J, Calderwood SB, Mylonakis E. *Cryptococcus neoformans* gene involved in mammalian pathogenesis identified by a *Caenorhabditis elegans* progeny-based approach. Infect Immun. 2005; 73:8219-8225.
31. Petzold EW, Himmelreich U, Mylonakis E, Rude T, Toffaletti D, Cox GM, Miller JL, Perfect JR. Characterization and regulation of the trehalose synthesis pathway and its importance in the pathogenicity of *Cryptococcus neoformans*. Infect Immun. 2006; 74:5877-5887.
32. Jong A, Wu CH, Chen HM, Luo F, Kwon-Chung KJ, Chang YC, Lamunyon CW, Plaas A, Huang SH. Identification and characterization of CPS1 as a hyaluronic acid synthase contributing to the pathogenesis of *Cryptococcus neoformans* infection. Eukaryot Cell. 2007; 6:1486-1496.
33. Reese AJ, Yoneda A, Breger JA, Beauvais A, Liu H, Griffith CL, Bose I, Kim MJ, Skau C, Yang S, Sefko JA, Osumi M, Latge JP, Mylonakis E, Doering TL. Loss of cell wall alpha(1-3) glucan affects *Cryptococcus neoformans* from ultrastructure to virulence. Mol Microbiol. 2007; 63:1385-1398.
34. Chayakulkeeree M, Sorrell TC, Siafakas AR, Wilson CF, Pantarat N, Gerik KJ, Boadle R, Djordjevic JT. Role and mechanism of phosphatidylinositol-specific phospholipase C in survival and virulence of *Cryptococcus neoformans*. Mol Microbiol. 2008; 69:809-826.
35. Lee A, Toffaletti DL, Tenor J, Soderblom EJ, Thompson JW, Moseley MA, Price M, Perfect JR. Survival defects of *Cryptococcus neoformans* mutants exposed to human cerebrospinal fluid result in attenuated virulence in an experimental model of meningitis. Infect Immun. 2010; 78:4213-4225.
36. Fan W, Idnurm A, Breger J, Mylonakis E, Heitman J. Eca1, a sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase, is involved in stress tolerance and virulence in *Cryptococcus neoformans*. Infect Immun. 2007; 75:3394-3405.
37. Morrow CA, Valkov E, Stamp A, Chow EW, Lee IR, Wronski A, Williams SJ, Hill JM, Djordjevic JT, Kappler U, Kobe B, Fraser JA. De novo GTP biosynthesis is critical for virulence of the fungal pathogen *Cryptococcus neoformans*. PLoS Pathog. 2012; 8:e1002957.
38. Cordeiro RA, Evangelista AJ, Serpa R, Marques FJ, Melo CV, Oliveira JS, Franco JD, Alencar LP, Bandeira TJ, Brilhante RS, Sidrim JJ, Rocha MF. Inhibition of heat shock protein 90 enhances the susceptibility to antifungals and reduces the virulence of *Cryptococcus neoformans*/*C. gattii* species complex. Microbiology. 2015. doi: 10.1099/mic.0.000222
39. Kozubowski L, Heitman J. Septins enforce morphogenetic events during sexual reproduction and contribute to virulence of *Cryptococcus neoformans*. Mol Microbiol. 2010; 75:658-675.
40. Lev S, Crossett B, Cha SY, Desmarini D, Li C, Chayakulkeeree M, Wilson CF, Williamson PR, Sorrell TC, Djordjevic JT. Identification of Aph1, a phosphate-regulated, secreted, and vacuolar acid phosphatase in *Cryptococcus neoformans*. MBio. 2014; 5:e01649-01614.
41. Chabrier-Rosello Y, Gerik KJ, Koselny K, DiDone L, Lodge JK, Krysan DJ. *Cryptococcus neoformans* phosphoinositide-dependent kinase 1 (PDK1) ortholog is required for stress tolerance and survival in murine phagocytes. Eukaryot Cell. 2013; 12:12-22.
42. Binder U, Maurer E, Lass-Flörl C. *Galleria mellonella*: An invertebrate model to study pathogenicity in correctly defined fungal species. Fungal Biol. 2015; 120:288-295.
43. Desalermos A, Tan X, Rajamuthiah R, Arvanitis M, Wang Y, Li D, Kourkoumpetis TK, Fuchs BB, Mylonakis E. A multi-host approach for the systematic analysis of virulence factors in *Cryptococcus neoformans*. J Infect Dis. 2015; 211:298-305.
44. Vu K, Gelli A. Astemizole and an analogue promote fungicidal activity of fluconazole against *Cryptococcus neoformans* var. *grubii* and *Cryptococcus gattii*. Med Mycol. 2010; 48:255-262.
45. Garcia-Rodas R, Casadevall A, Rodriguez-Tudela JL, Cuenca-Estrella M, Zaragoza O. *Cryptococcus neoformans* capsular enlargement and cellular gigantism during *Galleria mellonella* infection. PLoS One. 2011; 6:e24485.
46. Matsumoto Y, Ishii M, Ishii K, Miyaguchi W, Horie R, Inagaki Y, Hamamoto H, Tatematsu K, Uchino K, Tamura T, Sezutsu H, Sekimizu K. Transgenic silkworms expressing human insulin receptors for evaluation of therapeutically active insulin receptor agonists. Biochem

Biophys Res Commun. 2014; 455:159-164.
47. Inagaki Y, Matsumoto Y, Ishii M, Uchino K, Sezutsu H, Sekimizu K. Fluorescence imaging for a noninvasive *in vivo* toxicity-test using a transgenic silkworm expressing green fluorescent protein. Sci Rep. 2015; 5:11180.
48. Imamura M, Nakai J, Inoue S, Quan GX, Kanda T, Tamura T. Targeted gene expression using the GAL4/UAS system in the silkworm *Bombyx mori*. Genetics. 2003; 165:1329-1340.

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