Original Article

Infection of Chick Chorioallantoic Membrane (CAM) as a Model for the Pathogenesis of Cryptococcus gattii

Emmanuel Nnaemeka Nnadi¹, Ifeoma Bessie Enweani² and Grace Mebi Ayanbimpe³  

¹ Department of Microbiology, Faculty of Natural and Applied Sciences, Plateau State University  
² Department of Medical Laboratory Science, Faculty of health sciences and Technology, Nnamdi Azikiwe University  
³ Department of Microbiology, Faculty of Medical Sciences, University of Jos  

ABSTRACT

The use of embryonated egg as an alternative in the study of the pathogenesis of fungi is evolving. Although murine models are the “gold standard,” embryonated egg models are also used to screen determinants of virulence among fungi species. This study was aimed at determining the virulence potential of Cryptococcus gattii strains R265, R272, and EJB18, and Malassezia sympodialis using chorioallantoic membrane (CAM) of embryonated egg. At a concentration of 10⁷ cfu/ml, C. gattii R272 was more virulent than R265 in the egg model, while EJB18 had low virulence. The CAM model supported the growth of Malassezia sympodialis strain and induced the formation of hyphae. The formation of lesions by the organism and its re-isolation from CAM suggest that the model can be used for evaluating the virulence of C. gattii. Histopathology of CAM from both strains also revealed massive disruption of CAM. This study suggests that embryonated egg is a useful alternative tool to pre-screen Cryptococcus gattii strains to select strains for subsequent testing in murine models and could also be a potential medium for studying the hyphal growth in Malassezia species.

Key words: Cryptococcus gattii, embryonated egg, Malassezia, virulence

Introduction

Cryptococcosis is a clinically relevant disease that has a high disease burden; it is responsible for over 1 million infections, over 620,000 deaths annually, and one-third of AIDS-related deaths¹. Cryptococcosis is caused by Cryptococcus neoformans and Cryptococcus gattii. Cryptococcus gattii is a fungal pathogen that causes cryptococcosis in mainly immunocompetent individuals². Modern molecular techniques have aided in the understanding of this species, have led to the revision of the taxonomy of this pathogenic yeast, and have further enhanced our understanding of its ecology, epidemiology, and pathogenesis. C. gattii isolates have been reported to have four major linages using current genotyping tools (VGI, VGII, VGIII, and VGIV)³. A recent study by Hagen et al.⁴ proposed that C. gattii be split into five species, although a more recent study has suggested the use of Cryptococcus neoformans species complex” and “C. gattii species complex”⁵. Phylogenetic analysis suggests that C. gattii and C. neoformans diverged around 80-100 million years ago, and VGII is the ancestral molecular type to the C. gattii clade⁶,⁷. Both species have different ecological niches and vary in the patients they infect⁸. C. gattii is regarded as a primary pathogen that can infect immunocompetent individuals⁹, while C. neoformans is regarded as causing largely opportunistic infections.

Infection models are essential tools in investigating host-pathogen interaction, pathogenicity mechanisms, virulence attributes of pathogenic fungi, and therapeutic methods. Thus, in vivo models, mainly using laboratory rodents such as mice, have been developed for many microbial pathogens. These models have been critical for understanding host-pathogen interactions as well as for developing better therapeutic approaches¹⁰ and represent the current “gold standard” of in vivo virulence testing. The 3Rs for animal research are reduction, refinement, and replacement¹¹. Scientists prefer to use alternative models that do not pose legal problems. Models using C. elegans, G. mellonela, Drosophila, and silkworm¹².

Address for correspondence: Emmanuel Nnaemeka Nnadi  
Department of Microbiology, Plateau State University, Bokkos, Bokkos, Plateau NIGERIA  
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E-mail: eennadi@plasu.edu.ng
Several models have been used in the study of *C. gattii*\(^\text{15}\). Embryonated chicken eggs provide an alternative *in vivo* infection model that requires little specialized equipment, is easy to handle, and is available at lower costs than murine models. Embryonated bird eggs could provide an alternative model that would fulfill the demand for animal models. One advantage embryonated egg may have over invertebrate alternative models is that, being vertebrate, it would have closer immunity to mammals than invertebrates. This model has been successfully used in studying virulence determinants of viruses and bacteria\(^{16, 17}\). It has also been used successfully for studying the pathogenesis of some fungal pathogens such as *Candida*\(^{18, 19}\) and *Aspergillus fumigatus*\(^{20}\).

This method, however, has not been successfully used for *Cryptococcus*. Various laboratory studies have used embryonated egg for evaluating virulence *C. neoformans* without success, even at high concentration (10\(^8\) cells/egg)\(^{21}\). No study has reported the use of this method for *C. gattii*. This study, therefore, was aimed at using an embryonated egg model to study the virulence of *C. gattii*.

*Malassezia* hyphae are not well studied because they grow slowly *in vitro* and require specialized growth media that contains a lipid source\(^{22}\). Several media have been used to study the hyphal development in *Malassezia*. This study also set out to evaluate the suitability of embryonated egg in assessing the virulence of *M. sympodialis* and to investigate the induction of hyphae in the egg model.

**Materials and methods**

*Cryptococcus* strains were prepared for inoculation as described by Jacobsen et al.\(^{18}\). *C. gattii* strains R272, R265, and EJB1\(^{18}\) were used in this study. Additionally, *Malassezia sympodialis* strain ATCC42132 was used. For infection experiments, strains were sub-cultured once on YPD agar plates for 24 h at 37°C. A single colony was then inoculated into 20 ml liquid YPD and cultured for 12-14 h at 30°C with shaking at 200 rpm. Cells were harvested by centrifugation (3,000 g, 5 min) and washed twice with cold sterile phosphate buffered saline (PBS), pH 7.4. Cell concentration was determined using a haemocytometer. Suspensions were adjusted to the desired cell density with cold, sterile PBS. Inocula were maintained on ice and used within 1-2 h. The cell numbers in inocula were confirmed by plating serial dilutions on YPD agar plates. Colony forming units (cfu) were determined after 36 h incubation at 30°C. For virulence studies, the inoculum was adjusted to 10\(^5\) cfu/ml.

A hole was carefully drilled in the middle of the egg’s blunt pole using a dentist drill in a dark room to facilitate observation of depth of penetration. The hole penetrated the shell and shell membrane into the natural air space but did not disrupt the chorioallantoic membrane (CAM). This process was repeated for all eggs of the group. Removal of the natural air space was observed as a darkening of the air space. In parallel, air should flow into the second hole at the longitudinal site, causing lowering of the CAM and generation of an artificial air space. The artificial air space appeared as a sharp, oval, lighter area under the shell. Light in the room was turned on after artificial air space was created in all the eggs.

Inoculation was done by introduction of 100 µl of the infectious inoculum through the hole at the longitudinal site directly into the CAM using a sterile needle. Twenty (20) eggs were infected per strain. Both holes were sealed with transparent cellophane tape by gently rubbing it over the perforation, and the eggs were placed back into the incubator.

Survival was monitored for up to 7 days by candling the eggs once to twice daily in a dark room. Living embryos were identified by their tendency to move away from light; regardless of the speed at which embryos moved away from the light source, an embryo with movement was considered to be alive. Also, the presence of blood vessels that did not collapse indicated living embryos. Dead embryos were not active, and the blood vessels collapsed. At the end of the experiment, surviving embryos were killed by chilling on ice or freezing prior to autolclaving and disposal. Survival curves were prepared using Prism 6 (GraphPad) and statistically analyzed using the Log-rank (Mantel–Cox) test.

Embryonated eggs were inoculated with 10\(^5\) cfu/egg as described above. At given time points, viable embryos (defined by embryonic movement at candling) were humanely sacrificed by chilling the eggs on ice for 30-60 min. The egg surface was then disinfected with 70% ethanol. The shell was cut in half with sterile scissors. Approximately 0.5-1 g CAM (comprising 1/3 to 1/2 of the total CAM) was removed. Samples were homogenized in 2 ml sterile, cold PBS using a tissue homogenizer. Serial dilutions were made and plated onto YPD agar plates and incubated for 36 h prior to colony counting to determine cfu.

**Results**

To determine the suitability of the CAM model for studying virulence of *C. gattii*, 20 eggs were inoculated per strain using PBS as control. Inoculation of the CAM with *Cryptococcus gattii* strains led to mortality of the embryos (Fig. 1). R272 and R265 strains resulted in <25% mortality at day 7 when the experiment was terminated. Progressive mortality was observed for these isolates; however, for R265, mortality peaked and stopped on day 4 while for R272 progressive mortality was observed until day 7 when the experiment was terminated. *M. sympodialis* however, showed indistinguishable mortality when compared with the control group.

After 24 hours of inoculation of CAM with 10\(^5\) cfu/ml of...
fungi, lesions were observed on the CAM (Fig. 2). Macroscopic view of CAM after inoculation with *M. sympodialis* showed plaque formation following the production of nodes on day 3 of infection (Fig. 3). The nodes were carefully collected and squashed, stained with Calcoflour white, and viewed under × 40 objectives. The nodes were made of a mass of hyphae formed by *M. sympodialis* (Fig. 3b). This confirms the formation of hyphae on CAM by *M. sympodialis*. Histology of CAM following inoculation with *C. gattii* and PAS staining of CAM shows the presence of yeast cells and a massive disruption of CAM membrane (Fig. 4).

To determine dissemination of fungus, we attempted to re-isolate and quantify the *Cryptococcus neoformans* from CAM, liver, and brain. All of the strains could be isolated from the CAM, and the fungal burden was determined as colony forming unit (cfu/ml) in the CAM after 24, 48, and 72 hr after infection (Fig. 5 and 6). There was a transient reduction in the amount of fungi isolated from CAM on the second day. However, this was followed by a transient isolation of yeast for R272 and R265 from the brain (Fig. 6). EJB18 however did not disseminate to the brain. No organisms were isolated from the brain after the second day as it was cleared by the embryo. No organisms were isolated from the brain after the second day. The reason being that the organisms were possibly cleared by the embryo after the first day.

**Discussion**

Embryonated eggs can be infected via the CAM, the albumen, the yolk sack, or the allantoic cavity, or by direct injection into the embryo or major blood vessels. In this study, eggs were infected via the CAM for the following reasons: (i) the CAM is a thin, translucent membrane consisting of two epithelial layers separated by loose connective tissue\(^2\), (ii) pathogens applied to the outer layer of the CAM have to invade the outer epithelial layer to access nutrients, and (iii) sterile application of solutions onto the CAM is technically straightforward, and the required manipulation is well tolerated by the embryo\(^1\).

Previous studies using chicken embryos as alternative hosts vary in several technical aspects, thus hampering direct comparison of obtained results. The use of CAM has been successfully used for other fungi\(^18,20\). However, this model has not been used for *Cryptococcus*. Therefore, the study sought to characterize this model by using *Cryptococcus gattii* and *Malassezia sympodialis* (ATCC42132). The choice of CAM model is due to the large cost of maintaining murine models, the high technical requirements, and the necessity to address ethical issues. Thus, there was a need to seek alternative models that could suitably substitute for the murine model in studying pathogenicity of *Cryptococcus gattii*.

Different degrees of mortality rates were observed for the four test strains. Mortality has been confirmed to be dose-dependent in *Candida* model\(^18\). R272 and R265 strains were more virulent than EJB18 in the embryonated egg model. A transient isolation of yeast from the brain was observed for
R272 and R265, but not for EJB18. This may be suggestive of the fact that, as reported earlier, C. gattii affects the lungs more than the CNS. The fact that C. gattii can be isolated from the brain is an excellent advantage over Galleria mellonella, C. elegans, and other invertebrate models, where dissemination is very difficult to investigate.

The results obtained in this study however are different from those obtained in the murine model for these C. gattii isolates. In their study, Byrnes et al. showed avirulence for R272 while EJB18 was more virulent than R265.

Malassezia is able to exist in both yeast and mycelial forms, with the yeast being most commonly associated with normal skin. Less emphasis has been placed on the study of Malassezia and dermatophytes because they cause only superficial infections and are more amenable to topical treatment. Nevertheless, such infections are extremely common and can cause considerable distress to chronic sufferers.

Three major groups of filament-forming fungi commonly infect the keratinized and cornified layers of skin, nails, and hair—Candida albicans, the dermatophytes and the lipophilic Malassezia spp. Conidia and hyphae are observed in skin scrapings and in lesions caused by these fungi, which face the challenge of retaining a foothold on a surface that is being constantly shed. Candida spp. and Malassezia spp. are part of the commensal flora, and growth in the yeast form is generally asymptomatic and tolerated by the immune system.

Malassezia hyphae are not well studied because they grow slowly in vitro and require specialized growth media that contain a lipid source. In vivo, hyphae are observed only in

**Fig. 3.** Macroscopic examination of egg CAM infected with M. sympodialis
(a) White mass lesion observed on CAM.
(b) White mass viewed under x40 after staining with Calcoflour white showed hyphal formation.

**Fig. 4.** Histology of embryos infected on developmental day 1.
(a) CAM control shows integrity of CAM.
(b) Periodic acid-Schiff staining of the CAM shows the presence of yeast cells and massive disruption of CAM.
individuals with hyperactive sebaceous-gland activity, where the presence of excess sebum appears to be the inducer of morphogenesis\(^{23}\). Several groups have succeeded in inducing mycelial formation \textit{in vitro} using a variety of media such as cholesterol\(^{29}\), and through the use of synthetic media\(^{30}\).

Embryonated chicken eggs are a rich source of lipids and cholesterol\(^{31}\). The ability of embryonated egg to induce morphogenesis in \textit{Malassezia sympodialis} may be due to the high content of lipid in the egg, hence the embryonated egg model may be suitable in stimulating hyphal growth in \textit{Malassezia} and useful in studying the effect of hyphae in the pathogenesis of \textit{Malassezia}.

A variety of non-vertebrate hosts have been used to investigate the virulence of \textit{Cryptococcus}, including the use of amoebas, silkworm\(^{13}\), \textit{Acanthamoeba castellanii}\(^{32}\), and \textit{Dictyostelium discoideum}\(^{33}\). These models may not be applicable for the study of various species of \textit{Cryptococcus} as they respond differently to these models. Furthermore, in their study on the evaluation of CAM model for \textit{Candida albicans} and \textit{C. glabrata}, Jacobsen et al.\(^{18}\) observed a difference in virulence. In their study, \textit{C. glabrata} did not cause mortality in the CAM model although it was re-isolated from the CAM, hence making the CAM model unsuitable for the study of \textit{C. glabrata}.

Although the chicken model might yield false-negative results, it still provides a suitable screening tool to determine the virulence of large numbers of mutant strains in order to identify attenuated strains for subsequent testing in murine models. Furthermore, the chicken embryo model can be used as an alternative \textit{in vivo} system when specialized animal facilities are not available.

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\textbf{Conflicts of interest}

None.
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